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## Understanding the Chondrogenic Potential of Articular Chondrocytes

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# **UNDERSTANDING THE CHONDROGENIC POTENTIAL OF ARTICULAR CHONDROCYTES**

By

**Krishna Sarma**

A DISSERTATION

Presented to the Faculty of  
the University of Nebraska Graduate College  
in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy

Genetics, Cell Biology, & Anatomy  
Graduate Program

Under the Supervision of Professor Andrew T. Dudley

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## **Abstract**

# **Understanding the Chondrogenic Potential of Articular Chondrocytes**

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Articular cartilage is a smooth, visco-elastic, aneural, avascular tissue made of water, an exquisitely organized framework of proteoglycans, glycosaminoglycans, and collagen fibrils and articular chondrocytes. It's beautiful organization and composition provide it with the flexibility and strength to cover, protect and lubricate the ends of long bones in a diarthrodial joint. Cartilage homeostasis relies on articular chondrocytes to translate the mechanical forces of daily activity into efficient remodeling of the extracellular matrix. Age, joint injury, or other insulting factors can progressively incapacitate articular chondrocytes, resulting in cartilage lesions that devolve to degenerative joint disease. Therefore, the central idea explored in this dissertation is the changing chondrogenic potential of articular chondrocytes. In the first study, we asked if chondrogenic potential affects how primary articular chondrocytes respond to dynamic  $\text{Ca}^{2+}$  signaling, the primary signaling mediator of mechanotransduction during extracellular matrix remodeling. In the second study, we explored how age and culture conditions that alter chondrogenic potential influence the transcriptional profile of primary articular chondrocytes using in-depth RNA-sequencing technology. These studies highlight that the chondrogenic potential of articular chondrocytes, which is affected by age and the gradual changes in matrix composition, can be understood through dynamic signaling and transcriptional networks and enhanced through tissue engineering principles to improve upon the long-term efficacies of cartilage resurfacing procedures.

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**List of Abbreviations**

ACI	autologous chondrocyte implantation
AMIC	autologous matrix-induced chondrogenesis
BMP	bone morphogenetic protein
cDNA	complementary deoxyribonucleic acid
Col	collagen
DNA	deoxyribonucleic acid
ECM	extracellular matrix
FGF	fibroblast growth factor
IGF	insulin-like growth factor
IHh	indian hedgehog
MACI	matrix-induced autologous chondrocyte implantation
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T-cells
PCM	pericellular matrix
PTH	parathyroid hormone
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SOX	Sry-related HMG box
TGF $\beta$	transforming growth factor beta
TPM	transcript per kilobase million
VEGF	vascular endothelial growth factor
WNT	wingless/int-1



## **Chapter 1: Introduction**

## Introduction

One of the most beautiful and exquisite materials in nature is articular cartilage, which protects the end of long bones. Its smooth texture and pearly white gloss is reminiscent of porcelain, but unlike porcelain its coefficient of friction is nearly ten times lower than other naturally-occurring material and is among the lowest observed in the natural world<sup>1</sup>. Surprisingly, this smooth substance has the capacity to bear forces on the scale of MPa and up to ten times an individual's body weight<sup>1</sup>. The marvel of articular cartilage has captivated the attention of biologists, chemists, biophysicists, engineers, and surgeons alike.

The old anatomists' adage that "form fits function" holds very true in the case of articular cartilage. The stepwise development of synovial joints and subsequently the unique architecture of articular cartilage continues from *utero* into early adulthood. However, just as it begins to reach full maturity it must grapple with the decade-long demands of human activities such as walking, running, and recreational sports. As articular cartilage tries to maintain its structural framework, the continual "wear and tear," the onslaught of inflammatory and metabolic components, and age hinder the minimal reparative potential that the tissue has. Eventually, articular cartilage succumbs and gradually degenerates from small cartilage lesions to full thickness defects. The end stage of this process is a part of the condition we know as osteoarthritis.

Osteoarthritis is clinically defined as a deterioration of the entire synovial joint. The denuding of the articular cartilage is accompanied by the formation of osteophytes, subchondral bone remodeling, and synovial capsular inflammation; however, at the heart of the matter is the excruciating pain and immobility of individuals due to a gradually deteriorating articular surface. This degradation of articular cartilage is largely dependent

on the biology of the articular chondrocyte, as it is the sole cell found in the tissue and bears the burden of maintaining the structural framework of articular cartilage throughout an organism's lifetime. To understand the nature of articular cartilage degradation and the contributions of articular chondrocyte to this process it is important to consider the nature of articular cartilage and chondrocyte development.

## **Synovial joint development**

The origins of articular cartilage are mesenchymal in nature, as one of the critical events of skeletogenesis, the process whereby the limbs of an organism arise, is the condensation of mesenchymal stem cells<sup>2,3</sup>. This step developmentally precedes the commitment towards the various lineages, such as the chondrogenic or osteogenic lineage. There are three critical components of mesenchymal cell condensations that affect downstream developmental events: (1) cell-cell contacts, (2) cell proliferation, and (3) transcriptional profile of the participating cells (*Figure 1A*). Cell-cell contacts are mediated by N-CAM, N-Cadherin, and other ECM molecules and is critical for the proper aggregation of all the cells that will eventually give rise to the prechondrogenic cells<sup>2,3,4</sup>. For proper aggregation of cells and for the condensate to achieve a critical mass there must be enough cells, making cell proliferation a crucial event<sup>4</sup>. It is thought that this is mediated by elevated levels of cAMP and subsequent nuclear localization of protein kinase A (PKA)<sup>5</sup>. The final critical component of condensation is that there must be significant shifts in the transcriptional profile of the cells, such as upregulation of *Pax1*, *Pax9*, and *Sox9* and downregulation of BMP signaling, prior to termination of condensation and initiation of overt differentiation<sup>2,5</sup>.

While the condensation of mesenchymal stem cells dictates the timing and position of the future limb, it is a minority of mesenchymal cells, more commonly known as the

interzone, that ultimately give rise to the future synovial joint and its cartilage components (Figure 1A). Classical studies in the chick have illustrated the importance of the interzone by microsurgically dissecting away the small group of mesenchymal cells in the elbow joint, resulting in a fusion of the humerus, ulna, and radius<sup>6</sup>. This illustrates the early progenitor cells that eventually give rise to articular cartilage must come from the interzone; however, there are many other components of the synovial joint, such as the supporting ligaments and menisci that are also affected by the ablation of the interzone<sup>2,3,4</sup>. Recent studies have shown that a critical component to the downstream commitment of cells within the interzone is their transcriptional profile and spatial distribution throughout the interzone<sup>7</sup>. Two genes that play critical roles in the developing cartilage anlagen during early joint formation are *Matn-1* (matrillin-1), which is expressed by various cartilage anlagen except for articular cartilage, and *Dcx* (doublecortin), which is a microtubule-binding protein found in the articular chondrocytes of all synovial joints<sup>8-10</sup>. Lineage tracing studies using a *Matn-1* knock-in mouse to identify the subpopulation that gives rise to articular chondrocytes found that it was a group of cells at about E13.5 adjacent to the interzone that are both *Col II* positive and *Matn-1* negative, thereby distinguishing the origins of transient (growth plate) and permanent (articular) cartilage<sup>7,8</sup>. In contrast, a study looking at a *Dcx* knock-in mouse, which is a microtubule-binding protein, found that *Dcx* is expressed at E9.5 in osteochondral precursors and is maintained solely in interzone cells through E13.5, which is when the interzone appears, leading the authors to conclude that articular chondrocyte precursors can be traced to cells within the interzone<sup>7,9,10</sup>. Therefore, there remains some ambiguity as to the timing, location, and divergence of the precursors of articular chondrocytes from other members of the cartilage anlagen. More recently, there has been some clarification as to the specific fate of the cells within the interzone by performing gene expression analysis through isolation of RNA using laser-

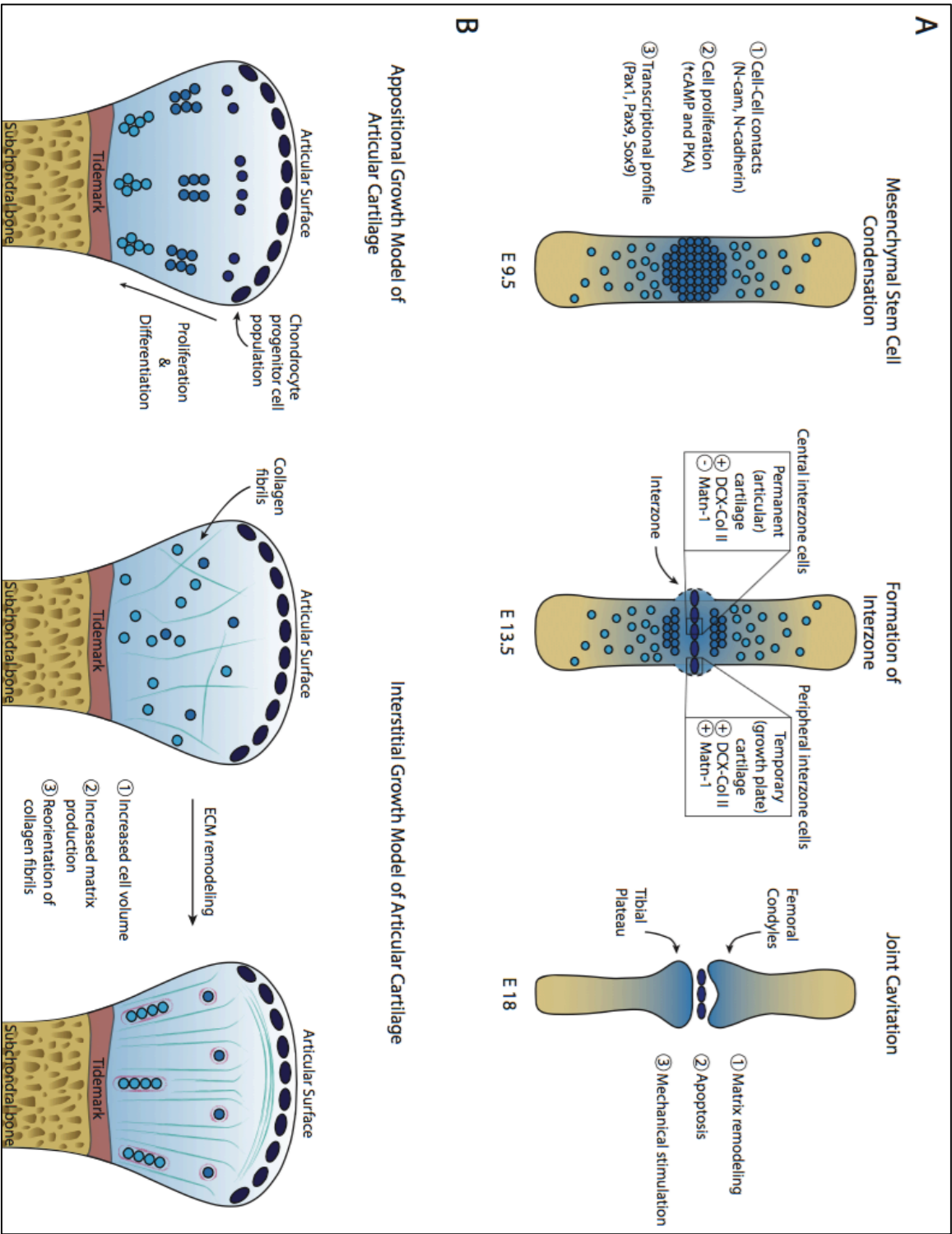
capture microdissection<sup>7,11</sup>. Utilizing *in vitro*, *in vivo*, and *ex vivo* methodologies on *Tgfb $\beta$ 2*<sup>Prx1KO</sup> mice, which have impaired synovial joint development, the authors found that expression of MCP-5, which is downregulated by TGF- $\beta$ 2, leads to improper delineation of the articular cartilage from the growth plate cartilage<sup>11</sup>. Furthermore, the study found that the distribution of cells within the interzone plays an important role in their downstream fate, as cells in the core of the zone differentially expressed genes important to articular cartilage formation, while cells in the periphery of the zone differentially expressed genes that mediate hypertrophy and growth plate cartilage formation<sup>7-11</sup>. There is now some clarity regarding the timing, location, and origin of the cells that will eventually give rise to articular chondrocytes during early joint formation; however, there remains pressing questions as to the molecular mechanisms driving the commitment of specific interzone cells towards a permanent cartilage lineage and repressing the commitment to an epiphyseal cartilage lineage.

The final stage of synovial joint formation that gives rise to its well-known morphology of two articulating long bones is joint cavitation<sup>12</sup>. Developmentally, cavitation is thought of as a solely apoptotic process; however, recent evidence suggests that local, macromolecular changes in matrix remodeling lead to a sequestration of cells within the interzone and formation of a joint cavity<sup>13,14</sup>. An elegant study using pharmacological and mutational analysis and an *in ovo* immobilization chick model that results in fusion of the developing cartilage anlagen illustrated that the mechano-dependent activation of the MEK-ERK 1/2 pathway is critical for interzone cells to remodel the hyaluronan-rich matrix for proper joint cavitation<sup>15</sup>. This study provided a beautiful mechanistic explanation for the importance of mechanical stimulation and matrix remodeling in joint cavitation, one of the final stages of synovial joint development. The coupling of mechanical stimulation and

matrix remodeling is a central principle that helps explain development and postnatal growth of articular cartilage as well<sup>16–21</sup>.

**Figure 1: Developmental program of synovial joints and differential growth models of post-natal articular cartilage.**

- A) There are three critical phases of synovial joint development: (1) Mesenchymal stem cell condensation (2) Interzone formation, and (3) Joint space cavitation. The initial event, cellular condensation, involves (1) tight cell-cell contacts, which is mediated by adhesion proteins N-CAM and N-Cadherin, (2) proliferation, mediated by signaling dynamics such as an increase in cAMP and nuclear localization of PKA, and (3) distinct transcriptional profiles, such as the expression of *Pax1*, *Pax9*, and *Sox9*. The interzone cells are considered the developmental progenitors of the cartilage anlagen. The specific cell population that gives rise to either the permanent (articular) and temporary (growth plate) cartilage elements is dictated by expression profile (presence or absence of *Matn-1* and *Dcx*) and spatial distribution (central or peripheral interzone cells). Lastly, cavitation is classically thought of as a solely apoptotic program; however, recent studies have shown that synovial joint cavitation involves mechanotransduction-mediated extracellular matrix remodeling and subsequent sequestration of distinct cell population.
- B) There are two prevailing postnatal articular cartilage growth models: (1) Appositional growth and (2) interstitial growth. The appositional growth model posits that there is a chondrocyte progenitor cell population in the superficial zone, which expresses *Prg4* and secretes the lubricating protein lubricin, that differentiates and proliferates to give rise to the cells of the remaining zones. This growth at the articular surface is thought to be concurrent with the advancing tidemark that separates the calcified articular cartilage from the subchondral bone. In contrast, the interstitial growth model claims that postnatally the chondrocytes are embedded within a random, disorganized extracellular matrix that is progressively remodeled through increased cell volume, increased matrix production, and reorientation of the collagen fibrils, with minimal cell proliferation.





## Articular Cartilage Development

Transient, or growth plate, cartilage consists of chondrocytes that undergo a developmental progression towards endochondral ossification<sup>22</sup>. This developmental progression is driven by a gradual differentiation of resting cells, which are thought of as the reservoir of growth plate progenitor cell population, to proliferative cells to pre-hypertrophic cells, and eventually hypertrophic cells that undergo apoptosis and endochondral ossification<sup>21,23–25</sup>. At skeletal maturity, the reservoir of resting cells is depleted and the growth plate closes and ends the growth of the skeletal anlagen<sup>24</sup>. This is in stark contrast to the permanent, or articular cartilage, which to date has not been shown to contain a progenitor cell population that would be able to differentiate and replenish the articular surface. This conventional view of articular cartilage being bereft of a progenitor cell population is being staunchly challenged by a plethora of studies.

The non-regenerative capacity of articular cartilage following an injury, as well as its avascular and aneural nature, is one of the main rationales for the claim that articular cartilage does not contain a progenitor stem cell population<sup>26</sup>. Furthermore, there is widespread dedifferentiation of articular chondrocytes isolated from adult articular cartilage, as evidenced by the downregulation of chondrogenic genes, onset of a hypertrophic phenotype, and altered morphology with minimal capacity to redifferentiate (personal observation). In stark contrast to these observations, however, is the identification of a group of totipotent articular chondrocytes among dedifferentiated human articular chondrocytes in monolayer culture<sup>27–29</sup>. This led to the search for a population of cells within the whole of adult articular cartilage that could serve as the source for articular cartilage growth. To test the hypothesis that articular cartilage growth occurs through an appositional mechanism from the articular surface, a group sought to turn their attention

to the superficial zone of articular cartilage. Indeed, they found a population of cells within the superficial zone that differentially adhered to fibronectin, had colony-forming potential, and responded to modulation of Notch signaling through alteration in differentiation potential<sup>25,27</sup>. Further support for the appositional growth model of articular cartilage has come recently from a group that has performed lineage tracing studies by generating a *Prg4* knock-in mouse model<sup>30</sup>. *Prg4* encodes for the articular surface lubrication protein, lubricin, which plays a critical role in generating the low coefficient of friction observed at the articulating surface of long bones<sup>31</sup>. By following the expression of *Prg4* over the course of a year the authors found that its expression propagated from the superficial layer at the first month to the full thickness of the articular cartilage at the end of the year<sup>28</sup>. The authors concluded that the cells present throughout the depth of the cartilage must be the progeny of the cells contained in the initial superficial layer. This model relies on the proliferation and subsequent differentiation of this chondrocyte progenitor cell population resulting in a greater cell density with development and growth; however, a closer examination of the data, especially at E17.5, would suggest that there is not an increase in cell density, but rather an increase in distance between the cells of the superficial layers and the deeper layers, possibly suggesting an increase in matrix biosynthesis. Nonetheless, there is growing evidence to suggest that there exists a population of progenitor cells within the superficial zone of articular cartilage that has the potential to expand and differentiate to allow for appositional growth (*Figure 1B*).

In contrast to the appositional growth model, other groups have posited that the postnatal growth of articular cartilage is due to extracellular matrix remodeling and changes in cell volume. Early studies looking at rabbit tibial plateaus ranging from newborns up to 6 weeks of age revealed that along with the development of the four major

zones of the mature articular cartilage collagen fibrils coordinately undergo development from randomly arranged fibrils to the more distinct orientations characteristic of the mature cartilage<sup>32</sup>. In similar opposition to the appositional growth model, a group recently used multiphoton microscopy on mice during a similar timeframe to ask if the postnatal growth of articular cartilage is attributed to chondrocyte volume, rather than proliferation<sup>33</sup>. The authors observed an increase in chondrocyte volume coupled with a decrease in cell density, due to increased matrix production, and concluded that articular cartilage may rely on morphological and architectural alterations, rather than biological changes, for postnatal growth (*Figure 1B*). The interstitial growth model, however, does not account for the presence of clonal columns of cells and the replenishment of lost cells through proliferation, which is thought to occur following mechanical compression to the articular surface<sup>34</sup>.

A combination of the two models may best explain the postnatal growth of articular cartilage. One of the possibilities is that embryonically you may get appositional growth through elevated proliferation of the chondrocyte progenitor population, however as the biochemical and mechanical microenvironment of the developing cartilage changes there will be a gradual shift in the developmental program from one of proliferation to one of extracellular matrix remodeling. This gradual shift, when combined with the development of the subchondral bone and advancing tidemark, may account for the thicker cartilage that is observed during skeletal immaturity (personal observations). It follows then that subchondral bone remodeling may outlast interstitial growth of articular cartilage which could account for the thinning of articular cartilage between postnatal growth and skeletal maturity<sup>35</sup>. Therefore, the rate of this shift in growth and

developmental program dictates the subsequent thickness and functional properties of the fully mature articular cartilage.

## **Form and Function of Articular Cartilage**

Up until this point our discussion has focused on the development of synovial joints and the cartilage that covers the articulating bones. In this section, we examine how the components and organization of articular cartilage are uniquely designed for it to bear up to ten times the body weight of the individual with smooth, frictionless motion<sup>1</sup>.

Articular cartilage is a heavily-hydrated tissue composed of variably distributed cells known as chondrocytes and a highly organized extra-cellular matrix that consists of collagens, proteoglycans, and non-collagenous proteins and glycoproteins<sup>36,37</sup>. The tissue is hierarchically organized with chondrocytes residing in single lacunae or clustering to form chondrons. These cellular units are embedded within matrices that vary in composition with distance from the cells. Taken further, the chondron units and associated matrices are organized into four distinct zones spanning from the articular surface to the subchondral bone. The four zones are superficial, transitional, radial, and deep (calcified) zone. These zones are distinguished by the composition and orientation of chondrocytes and extracellular matrices. The superficial zone consists of a thin layer of tightly-packed collagen that house flattened chondrocytes whose long axes lie parallel to the articular surface. The densely-packed collagen fibrils, with minimal intervening proteoglycans and glycosaminoglycans, serves as the primary physical barrier for the rest of the cartilage tissue. The elongated chondrocytes function to secrete the primary boundary lubricant, lubricin, and may serve as the progenitor cell population required for the appositional growth of articular cartilage<sup>31</sup>. The transitional zone contains spheroidal-shaped chondrocytes at a lower density than the superficial zone. While there may appear to be

fewer cells in the transitional zone its volume is greater than that of the superficial zone because of the presence of more proteoglycans. Relative to the superficial zone, the extracellular matrix of the transitional zone has a higher concentration of proteoglycans and glycosaminoglycans and a lower concentration of water and collagen fibrils, which are oriented obliquely to the articular surface<sup>36,37</sup>. This unique orientation of the collagen framework serves as a transition to the more radially orientated (perpendicular to the articular surface), cable-like, and sturdy collagen framework found in the aptly-named radial zone. The extracellular matrix of the radial zone contains the highest concentration of proteoglycans and the lowest concentrations of water, making it the most electrodynamic zone of articular cartilage. Firmly encased in the dynamic matrix of the radial zone are chondrocytes that are often organized into multi-chondron columns oriented perpendicular to the articular surface. The density of these chondral columns is the highest in the deep zone abutting the tidemark and subchondral bone. The tight interdigitation of articular cartilage and the subchondral bone is due to the passing of the radially-oriented collagen meshwork through the deep zone and into the tidemark, acting as a sort of anchor<sup>36,37</sup>. The organization of mature articular cartilage into four distinct zones is a gradual, developmental process that continues into early adulthood.

The extracellular matrix undergoes significant changes from birth to maturity due to the differing mechanical and biochemical environment from *in utero* to the postnatal synovial joint<sup>16-21</sup>. The functional component of the extracellular matrix consists of the interactions between collagen fibrils and the core of proteoglycans, the electrostatic interactions between the side chains of these proteoglycans with water and the counterbalancing ions, and the interactions between the matrix component and articular chondrocytes<sup>36,37</sup>. While at birth the distribution of the fibrillar and non-fibrillar collagens

are unorganized, with maturation, there is greater crosslinking and a distinct orientation to the fibrils<sup>32,33</sup>. It is still unknown whether these changes in collagen fibrils then initiate the changes in proteoglycan organization or vice-versa; however, like the collagen fibrils, there is significant modification to the number and nature of the sulfate chains attached to the proteoglycans during the weeks following birth, which alters the electrostatic interactions and ionic environment of the tissue<sup>38, 39–41</sup>. In addition to matrix remodeling, differential gene expression between the four zones also gives rise to zonal variations in architecture<sup>42,43</sup>. Microarray analysis of each zone indicates that the superficial contains the highest number of differentially-expressed genes and the middle zone contains the lowest number of differentially-expressed genes<sup>39</sup>. This finding is corroborated by the unique morphology of superficial zone chondrocytes, which helps to explain the decreased expression of type II collagen relative to the other zones, and the paucity of proteoglycans in this zone. These zonal variations in matrix architecture, matrix composition, and gene expression lead to differing mechanical and functional properties throughout the full-thickness of articular cartilage; however, there is an additional layer of anisotropy influencing articular chondrocyte biology.

The extracellular matrix surrounding the chondral columns of the radial zone, as outlined above, are further hierarchically-organized. An individual chondrocyte within the chondral column is surrounded by matrices that differ in their composition of collagen and proteoglycans, and thereby serve unique functions as signaling conduits<sup>44</sup>. The pericellular matrix, which envelopes the chondrocytes, is visualized as a thin film covering the plasma membrane. The chondrocyte and its surrounding pericellular matrix make up a single chondron unit<sup>45</sup>. It is through changes in the pericellular matrix that the chondrocyte detects and responds to mechanical, electrical, and osmotic stimuli<sup>46</sup>. The

predominant collagen of the pericellular matrix is collagen VI and it contains minimal number of proteoglycans and glycosaminoglycans relative to the other matrices. In between the pericellular matrices of two neighboring chondrons is the territorial matrix. The territorial matrix contains a higher concentration of fibrillar collagens, such as collagen II and IX, and serves as a common extracellular space between chondrons of a column, allowing for a common signaling conduit<sup>47</sup>. Separating the columns of the radial zone and making up the largest volume is the interterritorial matrix. This region contains the highest number of proteoglycans and collagens, and as such undergoes the biggest changes during physicochemical stimuli<sup>47</sup>. The organization and composition of the matrices in this manner serve two functions: (1) Protect chondrocytes from excessive forces and (2) act as a template for future remodeling purposes. With each loading event, there is exudation of water from the interterritorial matrix leading to activation of mechano-osmosensitive receptors; however, there is a certain threshold that must be reached for the signal to pass through all the matrices down to the encased chondrocyte. Upon removal of the load, the water is imbibed back into the matrix and once again triggers the activation of mechano-osmosensitive receptors<sup>36,37</sup>. Therefore, this organization allows for protection by allowing the chondrocytes to remain metabolically inert, which is critical due to the paucity of nutrients available to chondrocytes, and respond to stimuli only upon adequate deformation of all matrix regions<sup>36,37</sup>. As a result, not every loading event is perceived by the chondrocyte, however, the gradual deformation of the surrounding matrices allows the chondrocyte to gradually remodel the matrix over a long period. It is unknown as to the mechanisms by which the cells can efficiently detect small changes in the extracellular matrix of the different regions and adequately remodel them from one loading event to the next. It is likely that uncovering this fundamental mechanism will help explain the origins of articular cartilage degeneration. Taken together, the beautiful architecture of articular

cartilage from the four zones to the rings of matrices provide an extraordinarily unique environment for the articular chondrocyte.

### **Cartilage homeostasis through chondrocyte-mediated matrix remodeling**

Articular chondrocytes develop coordinately with the surrounding extracellular matrix through the establishment of a feedback loop between the remodeling extracellular matrix of articular cartilage and the differentiating chondrocyte progenitor cell population. The introduction of novel mechanical cues, the remodeling of the extracellular matrix, and the availability of ligands are interdependent factors that shape the signals presented to the developing articular chondrocytes, which then secrete factors to remodel its environment. The two major signaling cues to articular chondrocytes are mechanical stimuli and osmotic stimuli, specifically ion flux<sup>48, 49,50</sup>. In this section, we examine how mechanical forces set the feedback loop in motion and then examine the role of osmotic stimuli, specifically calcium signaling, in maintaining this regulatory mechanism for cartilage homeostasis.

The mechanical forces that initiate matrix remodeling during development also initiate novel signaling mechanisms by which chondrocytes can sense the microenvironment<sup>15</sup>. Mechanical forces signal to chondrocytes by releasing various cytokines and soluble factors that are trapped in the ECM, by activating cell surface receptors, or by initiating stretch-activated channels<sup>51</sup>. It is known that FGF2 bound to perlecan of the PCM is released upon mechanotransduction to signal into chondrocytes<sup>52</sup>. The subsequent cellular response is to remodel the ECM, which progressively alters the bioavailability of the signaling molecules during future stimuli. The integrin signaling pathway is well characterized as the pathway through which chondrocytes detect alterations in the PCM<sup>53,54,55</sup>. Specifically, cells respond to



mechanical cyclic pressurization through integrin  $\alpha V\beta 1$  receptor for downstream effects<sup>56</sup>. In addition to matrix deformation, fibronectin fragments, which are deleterious byproducts of cartilage breakdown, have been shown to signal through integrin receptors to elicit downstream MAPK signaling in chondrocytes<sup>57</sup>. Activation of these signaling pathways leads to progressive breakdown of the matrix further releasing a bevy of pro-inflammatory cytokines<sup>54</sup>. The chondrocytes, however, respond to alternative cytokines to counteract these catabolic and pro-inflammatory effects, such as IL-6-mediated upregulation of TGF- $\beta 1$  and integrin-mediated autocrine signaling of IL-4, both of which are chondroprotective factors for human articular chondrocytes<sup>58–60</sup>.

Receptors/channels that have garnered considerable attention for their role in conveying changes in cartilage ECM are Piezo receptors, which are thought to play pivotal roles in conferring mechanosensitivity to articular chondrocytes<sup>61</sup>. It follows then that these studies support the idea that the mechanical forces driving development of the extracellular matrix influences the signaling dynamics of articular chondrocytes as well; but an important component of establishing the feedback loop is articular chondrocyte-mediated remodeling of the extracellular matrix.

Remodeling the ECM is a dynamic process that entails breaking down the compromised matrix and replacing it with newly synthesized matrix components<sup>62</sup>. Cartilage homeostasis is dependent on the balance between matrix degrading and matrix rebuilding processes<sup>63</sup>. This balance is achieved through the concerted efforts of both anabolic and catabolic processes of articular chondrocytes<sup>64</sup>. Following exposure to stimuli, chondrocytes employ a variety of matrix metalloproteinases (MMPs), also known as collagenases, to break down the damaged ECM. Appropriate breakdown of the ECM requires precise spatial and temporal expression of specific MMPs to maintain cartilage

homeostasis as evidenced by the attenuation of PTOA in an *MMP-13* KO mouse model<sup>65</sup>. Following appropriate clearance of the damaged ECM, the chondrocytes secrete critical components of the matrices, such as proteoglycans, aggrecan, and the various fibrillar collagens<sup>66</sup>. The chondrocytes maintain themselves in the chondron units surrounded by territorial and interterritorial matrices for many years by secreting these critical components in immature forms<sup>62</sup>. This serves two purposes: (1) It stratifies the components among the different matrices and (2) distinguishes the physical properties of the different matrices surrounding the chondrocytes. These two properties of matrix production are dependent on the appropriate maturation of the components as they diffuse away from the chondrocytes, as well as the interaction between all components of the matrix. The physical property of the tissue is dependent on chondrocytes being able to synthesize aggrecan that will bind to link protein and hyaluronan and interdigitate within the collagen meshwork<sup>67,68</sup>. These interactions then create a negatively-charged environment that attracts both water and counterions that give the tissue its physicochemical properties.

Once chondrocytes are stimulated by changes in the ECM this iterative process of catabolic and anabolic processes is set in motion to continually maintain the integrity of the tissue. It follows then that for this to be a highly efficacious process the cells must perfectly orchestrate the interpretation of the changes in ECM with the appropriate breakdown and rebuilding of the ECM, thereby giving rise to a highly interdependent process. What remains unknown is if the development of mechanosensitivity is temporally linked to the maturation of the extracellular matrix? Also, if there is a delay in development of mechanosensitivity or a complete absence of mechanical stimulation/forces how does it affect the transcriptional dynamics of articular

chondrocytes? Additionally, does the absence of appropriate mechanical cues *in utero* and during the critical weeks postnatally compromise articular cartilage growth, thereby setting up an environment that is more susceptible to degeneration? The answers to these questions would provide mechanistic details as to how mechanical forces influence matrix remodeling, chondrocyte biology, and articular cartilage development, growth, and homeostasis.

### **Calcium signaling drives cartilage homeostasis**

The tight interaction between proteoglycans, water, and counterions creates an environment in which changes in matrix composition and organization drastically alter the water content and ionic milieu of the tissue, which in turn lead to alterations in osmotic forces<sup>49,62,69</sup>. These osmotic forces result from local changes in fluid flow and ion flux as water exudes from the tissue during joint loading and imbibes the tissue upon relaxation<sup>36,37</sup> (*Figure 2*). This dynamic fluid flow in the presence of negatively-charged proteoglycans and counterions results in streaming potentials that have been shown to increase the biosynthetic activity of chondrocytes<sup>70,71</sup>. Recently, attention has shifted to the effect of ion channels on chondrocyte biology and cartilage homeostasis<sup>72</sup>. The electrical milieu of articular cartilage provides credence to the importance of ion channels in the biosynthetic activity, with further support coming from groups that have established the differential regulation of chondrocyte membrane potential in normal and diseased cartilage<sup>73</sup>. One cation that plays a critical role in cartilage development and homeostasis is  $\text{Ca}^{2+}$  signaling.

Calcium signaling plays a critical role in many facets of skeletal development, especially chondrocytes<sup>23,74–77</sup>. A tissue-specific deletion of the extracellular calcium sensing receptor in chondrocytes resulted in embryonic-lethality by E13, while

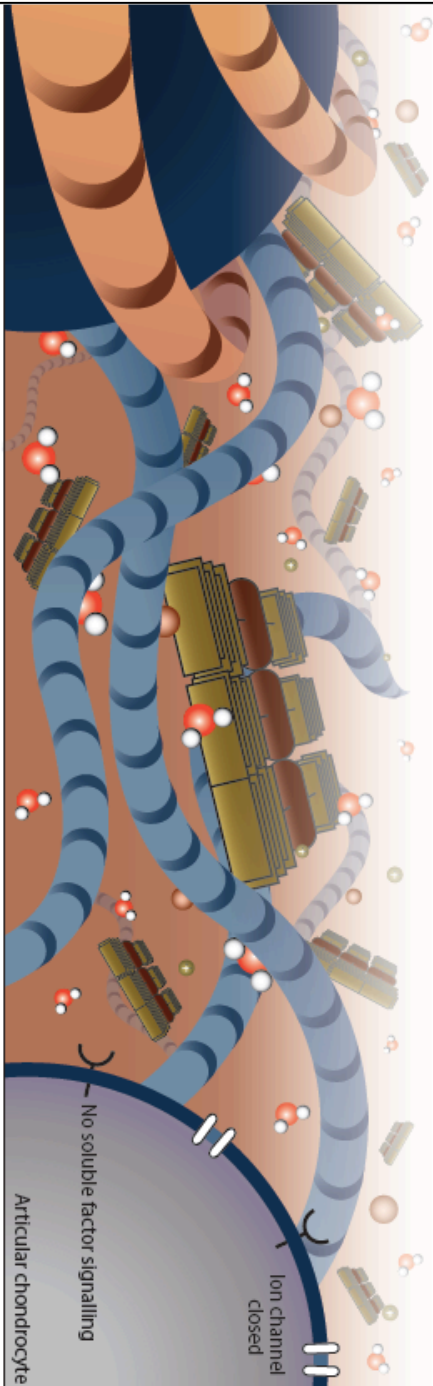
conditional deletion later in development resulted in widespread skeletal defects, implicating improper development of the cartilage anlagen<sup>78</sup>. In studying chondrogenesis, studies show that the expression of specific ion channels in progenitor cell populations is critical for regulating calcium signaling. For example, tracheal cartilage relies on the expression of  $Ca_v3.2$  T-type calcium channels for NFAT-mediated upregulation of the chondrogenic factor, *Sox 9*; however, it is not only calcium channels that drive chondrogenesis, as studies in primary micro-mass cell cultures from embryonic chick limb buds demonstrate that the expression of potassium channels also regulate calcium transients that are important for committing progenitor cells down the chondrocyte lineage<sup>79, 80</sup>. Similarly, inhibitor studies of calcium channels have abrogated the chondrogenic effects of hydrostatic pressure on mesenchymal stem cells<sup>76,81,82</sup>. Therefore, it is evident that in concert with mechanical forces calcium signaling plays an important role in the development of articular cartilage<sup>83,84</sup>. However, an area that remains unexplored is how the reorientation of collagen fibrils and the modifications to proteoglycans affect calcium signaling. It is possible that with the dynamic maturation of the collagen and proteoglycan network during the first weeks following birth there is a commensurate change in capacity for calcium transients and calcium signaling<sup>40,41</sup>. It begs the question then if these initial transients could be responsible for the potential transcriptional shift from a proliferative developmental program to a matrix-synthesizing program, thereby supporting the combination of the appositional and interstitial growth model to explain postnatal articular cartilage changes. The answers to these questions would have far-reaching implications because it would provide a mechanistic explanation for the interdependence of extracellular matrix remodeling and the changing transcriptional profile of articular chondrocytes in development, growth, homeostasis, and disease.

Just as in growth and development, mechanical and osmotic forces stimulate articular chondrocytes to drive cartilage homeostasis<sup>50,72,85</sup>. It is well known that both mechanical and osmotic forces, in the form of fluid flow, result in  $\text{Ca}^{2+}$  transients and waves that are propagated to multiple articular chondrocytes<sup>86–88</sup>. Several groups have suggested that these  $\text{Ca}^{2+}$  waves are transduced through an ATP-dependent purinergic pathway in cilia dependent and independent manner<sup>86,89</sup>. Taken further, it has been shown that changes in intracellular  $\text{Ca}^{2+}$  dynamics, instead of merely fluid flow, leads to the biosynthetic activity of chondrocytes<sup>88</sup>. Additionally, mechanical cues activate  $\text{Ca}^{2+}$  signaling by releasing cytokines, such as IL-1, and soluble factors, such as IGF, trapped in the extracellular matrix<sup>90,91</sup>. Similarly, many groups have established that osmotic stress/loading activates  $\text{Ca}^{2+}$  signaling to not only induce gene expression changes, but also remodel the cytoskeleton, which affects chondrocytes by modifying its subsequent response to stimuli<sup>84,92,93</sup>. Conversely, other studies have illustrated that  $\text{Ca}^{2+}$  signaling plays a key role in mediating mechanically induced chondrocyte death<sup>94,95</sup>. These studies demonstrate that articular chondrocytes mediate cartilage development, growth, and homeostasis through dynamic  $\text{Ca}^{2+}$  signaling. What is unknown, however, is if the channels identified in mature articular chondrocytes are also present in the developing cartilage. Differential expression of these channels and receptors across different age groups would point to two states that differ in mechanosensitivity and ability to respond to calcium signaling, which has been shown to drive cartilage homeostasis and degeneration.

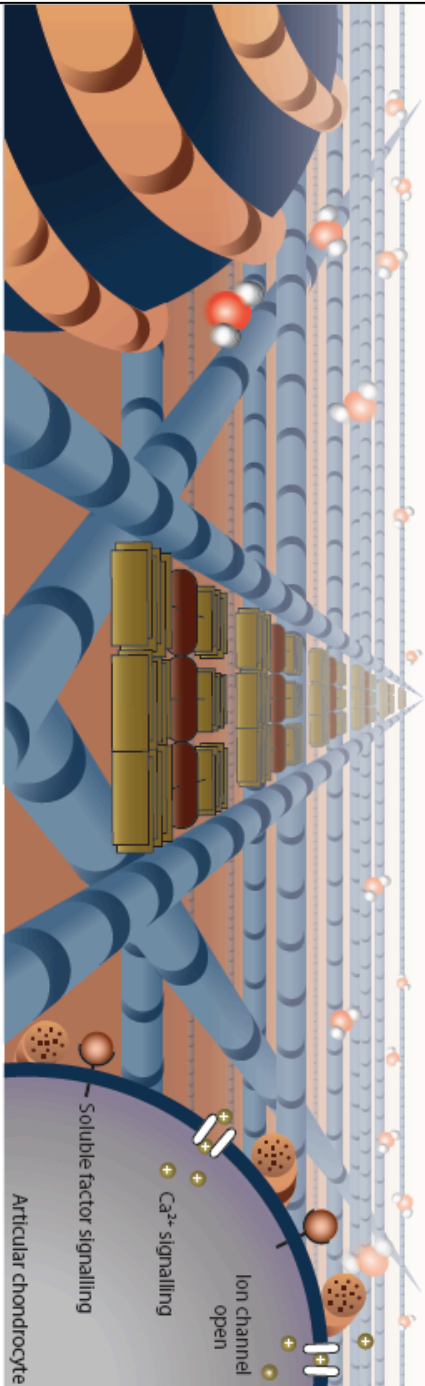
**Figure 2: Articular chondrocyte biology and changes in extracellular milieu during joint motion.**

Articular cartilage is an aqueous tissue with a dense matrix composed of tightly-packed collagen fibrils and proteoglycans, such as aggrecan, studded with sulfate groups that confer a negative charge to the tissue. This negative charge attracts counterions such as  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  giving rise to an electric field within the tissue. In addition to attracting counterions, the proteoglycans trap soluble factors and serve as a signaling reservoir. When combined with Collagens II, IX, and XI, the soup of ions, soluble factors, and proteoglycans make up the territorial matrix surrounding the pericellular matrices of articular chondrocytes. The primary constituent of pericellular matrices is collagen VI, a non-fibrillar collagen, and the primary function of this matrix is to serve as signaling conduit for mechanical and chemical stimuli to the articular chondrocyte it encases. Upon mechanical stimulation, water exudation results in a sharp increase in the osmolarity, influx of ions, and a release of soluble factors and ligands to the receptors on the cells. Concurrently, there is an increase in the tautness of the collagen fibrils which is the primary mechanism by which the tissue can efficiently resist compression. Taken together, the articular chondrocyte dynamically perceives these changes in the tissue through cell surface receptors and mechano- and osmosensitive ion channels and responds by secreting proteases to degrade the damaged matrix and matrix molecules to remodel the damaged tissue. Upon release of the mechanical stimulation, the water imbibes the tissue once again to rapidly change the osmolarity, flux of ions, and tension on the collagen framework. Articular chondrocytes respond to the cyclical nature of mechanical loading and unloading through remodeling of the extracellular matrix, which progressively decreases in efficiency; thereby affecting the signaling conveyed to the chondrocytes. Over time, chondrocytes respond to this altered stimulus through ineffective remodeling, resulting in cartilage lesions.

Unloaded Joint



Loaded Joint



- Soluble factors (GFs, FGFs, TGF-β)
- H<sub>2</sub>O
- Ca<sup>2+</sup>
- Proteoglycans
- Territorial matrix (Collagen II & IX)
- Pericellular matrix (Collagen VI)
- Cell surface receptor

## Orthopedic surgical approach to articular cartilage restoration

In the previous section, we discussed the importance of the articular chondrocyte in extracellular matrix remodeling to maintain cartilage homeostasis<sup>96</sup>. This process gradually deteriorates with age and trauma, resulting in cartilage lesions. These defects in the articular surface are part of a constellation of symptoms that when combined with other joint pathologies give rise to osteoarthritis. The biology of cartilage degeneration and the inflammatory component that eventually gives rise to osteoarthritis is extensively reviewed in the literature<sup>58,85,97–105</sup>. Therefore, we continue our discussion by examining how biologists, engineers, and surgeons collaborate to rectify the problem of full-thickness cartilage defects in the young patient.

There is a high incidence of traumatic cartilage lesions due to sports injuries and other recreational activities in the active, younger patient population<sup>106</sup>. At the time of surgery to repair the primary defect, such as a ligament or meniscal tear, there is a small cartilage lesion as small as 1cm<sup>2</sup>; however, the altered joint biology changes the mechanical loading of the articular surface, which can incite the lesion to grow up to 12cm<sup>2</sup> in size<sup>107–112</sup>. These cartilage lesions, if left untreated, will give rise to unbearable joint pain and fulminant osteoarthritis, necessitating total joint replacement. Therefore, the critical unmet need at the junction of articular cartilage biology and orthopedic surgery is the progressive decline in regenerative capacity of articular cartilage and the poor long-term efficacy of surgical procedure to resurface diarthrodial joints.

The regenerative capacity of articular cartilage is minimal due to its complex organization that requires highly specialized cells such as articular chondrocytes and its avascular and aneural nature. The limited regeneration that occurs is a result of infiltration of bone marrow elements upon penetration of the subchondral bone and often results in



a marrow clot. This biological phenomenon forms the basis of microfracture surgery, wherein the surgeon debrides the cartilage lesion and creates clean margins for the bone marrow elements that seep through the fractures introduced to the underlying subchondral bone (*Figure 3*). Microfracture surgery is often recommended for joints undergoing heavy load due to the individual's lifestyle and smaller lesions ( $\leq 2\text{cm}^2$ )<sup>113,114</sup>. Over the last twenty years, attention has turned to using biologics and tissue engineered biomaterials to fill larger cartilage lesions ( $\geq 10\text{cm}^2$ ). The most common surgical procedures employed to resurface the joint in the case of larger lesions are autologous chondrocyte implantation (ACI) and matrix-assisted autologous chondrocyte implantation (MACI) (*Figure 3*). ACI is a two-part procedure, first cartilage is harvested from a non-weight bearing surface and digested with bacterial endopeptidases releasing chondrocytes that are expanded *in vitro*. Second, upon sufficient expansion the cells are re-implanted into the debrided defect site and covered with a periosteal flap<sup>107,109,115</sup>. MACI is like ACI, however, prior to implantation the chondrocytes are embedded in a 3D scaffold, which is then implanted into the defect site and covered with a periosteal flap<sup>115</sup>. There are two major issues with these procedures: (1) during *in vitro* expansion the chondrocytes dedifferentiate and eventually produce fibrocartilage, which is biochemically and biomechanically inferior to the native hyaline cartilage, and (2) there is a high rate of hypertrophy in the implantations. These drawbacks are addressed by examining the cellular and scaffold component of the procedures.

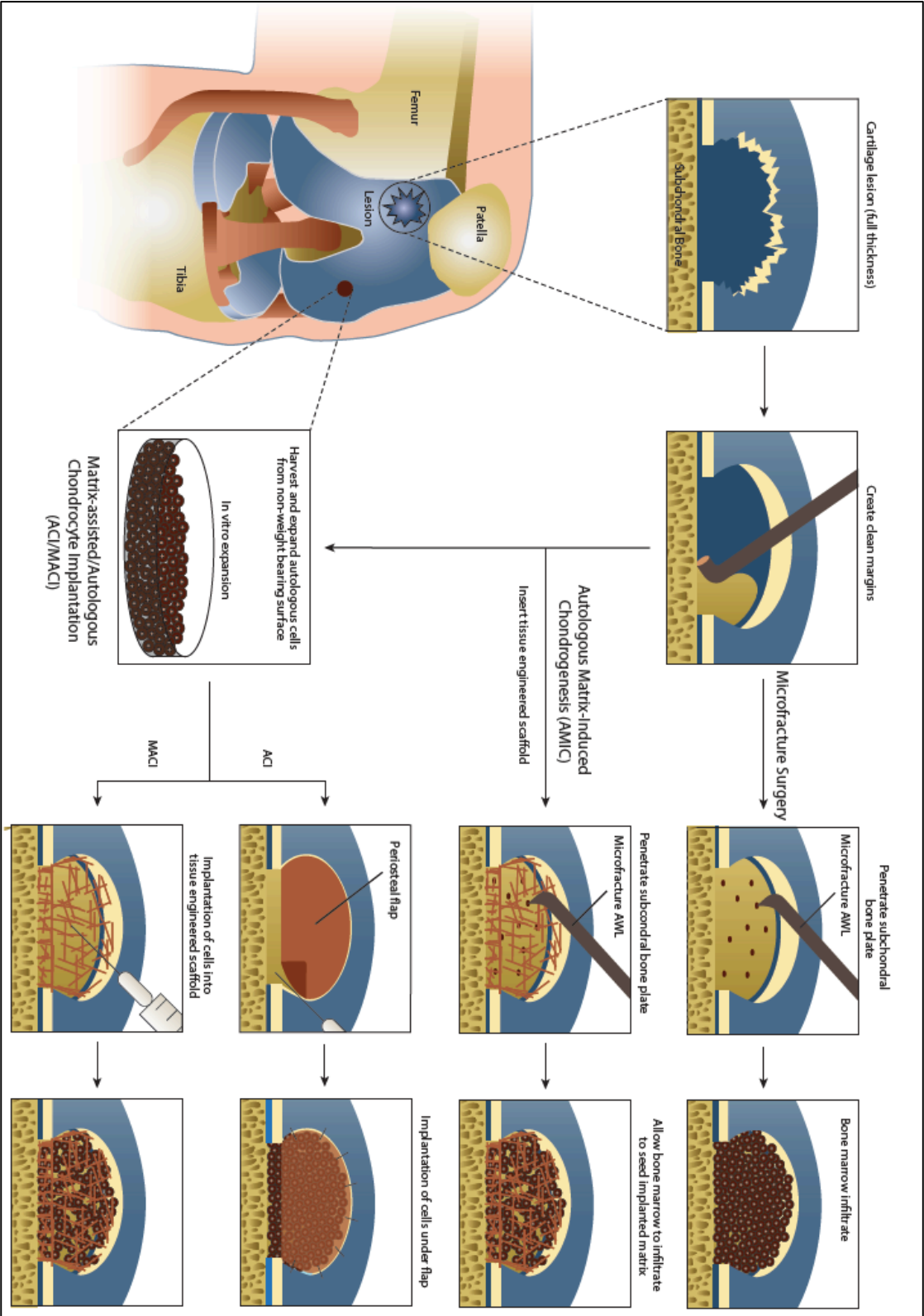
Adequate restoration of full-thickness cartilage lesions requires healthy chondrocytes to fill the defect site with a hyaline-like matrix that can withstand the forces that come with daily activity. It follows then that the critical element for long-term efficacy of these surgical procedures is the cellular potency of the chondrocytes injected into the

defect site. Recently, a group observed that patients undergoing ACI had higher knee function scores when treated with cells that have higher viability and a higher expression of CD44, type II collagen, and aggrecan; further extending the argument that cell quality plays a critical role in long-term outcomes for ACI<sup>116</sup>. Considering this, how do we increase the cellular potency of the injected chondrocytes? A recent study explored the idea of using cadaveric juvenile human articular chondrocytes instead of a patient's own cells<sup>117</sup>. The authors found that chondrocytes from a younger patient had up to a 700-fold higher expression of type II and IX collagen and almost a 100-fold higher production of proteoglycans than chondrocytes from older patients<sup>117</sup>. This raises a fundamental question about the role of aging in articular chondrocytes: How do chondrocytes from a younger patient differ from those of an older patient whose cells may have endured various surgical procedures and other joint pathologies? Another means to enhance the cellular potency of the implanted chondrocytes is the supplementation of a scaffold during the resurfacing procedure. Variants on the collagen-based scaffold for the autologous cells, MACI, are chitosan-based and hyaluronic acid-based scaffolds that have given mixed results as to their long-term efficacy<sup>118</sup>. In contrast to implanting a scaffold studded with cells, another approach has been to implant a scaffold into the defect site concurrent with microfracture surgery. This procedure is known as autologous matrix induced chondrogenesis (AMIC) and has the advantage of being a one-step procedure and allows for bone marrow elements to infiltrate and secrete a matrix on to the collagen scaffold, thereby minimizing the potential for the formation of a pure fibrocartilage clot<sup>119</sup> (*Figure 3*). Once again, these studies raise interesting questions about interaction between the implanted chondrocytes and the scaffold/environment of the joint in question. Lastly, a novel approach to addressing these issues is to utilize synthetic biology to engineer cells that respond dynamically to a changing environment. Recently, a group has been able to

engineer the genome of stem cells to modulate the response to pro-inflammatory cytokines, such as IL-1 and TNF- $\alpha$ , which are found in synovial joints and traumatic cartilage lesions<sup>120</sup>. The question remains, however, if the cells being implanted have the potential to produce a long-lasting biomechanically stable matrix.

**Figure 3: Orthopedic surgery and tissue engineering strategies to resurface the ends of the long bones.**

There are four major cartilage resurfacing surgical procedures: Microfracture surgery, Autologous Matrix-induced Chondrogenesis (AMIC), and Matrix-assisted/Autologous Chondrocyte Implantation (MACI/ACI). The first step in the cartilage resurfacing procedures is to debride the lesion site and create clean margins for the clot/implantation. In microfracture surgery, the principle is to create small lesions penetrating the subchondral bone plate with a microfracture awl to allow bone marrow elements to infiltrate the lesion site and form a fibrin clot to fill the defect site. ACI/MACI is a two-phase surgical procedure, the first step is to harvest and expand autologous chondrocytes from a non-weight-bearing surface for *in vitro* expansion. Following sufficient expansion, the cells are either injected underneath a periosteal flap that overlies the defect site in the case of ACI, or injected into a three-dimensional scaffold, which is modifiable in its composition, in the case of MACI. AMIC is a hybrid of microfracture surgery and MACI because the goal of the procedure is to allow the microfracture-induced bone marrow elements to infiltrate a tissue-engineered scaffold that is placed into the defect site. In all of these procedures the composition of the scaffold, the origin of cells that are injected into the defect site, and the composition of the flap that covers the defect site are modifiable elements that are under investigation for improvements in long-term efficacy.



## **The chondrogenic potential of articular chondrocytes**

The articular chondrocyte plays a crucial role in the development, growth, maintenance, and destruction of articular cartilage. In this chapter, I introduced the interdependence of articular cartilage development and growth and the proliferation, differentiation, and homeostasis of articular chondrocytes. Additionally, the importance of articular chondrocyte biology is exemplified by efforts to utilize them in cartilage resurfacing procedures. The rising incidence of osteoarthritis and the poor long term efficacy of these procedures illustrate our shortcomings in understanding the spectrum of articular chondrocyte biology ranging from health to disease.

In this dissertation, I explore the chondrogenic potential of articular chondrocytes in the context of  $\text{Ca}^{2+}$  signaling. During development, it is understood that there is a drastic change in matrix composition and organization driven by mechanical cues, which also activate  $\text{Ca}^{2+}$  signaling that is important for enhancing chondrogenesis of mesenchymal stem cells<sup>50,75–77,83,121</sup>. Therefore, the chondrogenic potential of articular chondrocytes in the developing cartilage may be dependent on the active remodeling of collagen fibrils and proteoglycans, which control the electrochemical properties of the tissue. While many studies have explored different factors that can enhance chondrogenic potential, the reciprocal relationship of the chondrogenic potential of articular chondrocytes affecting the response to stimuli remains unexplored. We find that the chondrogenic potential of articular chondrocytes dictates how the cells respond to  $\text{Ca}^{2+}$  signaling. These results provide support for asking how the reciprocal relationship between calcium signaling and chondrogenic potential drives development of articular cartilage, maintains cartilage homeostasis, and alters the regenerative capacity of articular chondrocytes.

## **Chapter 2: Understanding chondrogenic potential through Ca<sup>2+</sup> signaling**

## Introduction

Articular cartilage, the smooth, visco-elastic substance that covers the ends of long bones, protects and lubricates diarthrodial joints by evenly distributing forces across joint surfaces<sup>36</sup>. The denuding of articular cartilage gives way to degenerative arthritis, which affects nearly 40 million Americans<sup>114</sup>. The most common surgical procedure employed to resurface the joint is autologous chondrocyte implantation (ACI)<sup>109,122,123</sup>. During ACI, cartilage is harvested from a non-weight bearing surface and enzymatically digested to release articular chondrocytes<sup>109,122,123</sup>. The isolated cells are expanded *in vitro*, implanted into the defect site, and covered with a periosteal flap<sup>109,122,123</sup>. A major drawback to this procedure, however, is that chondrocyte dedifferentiation during *in vitro* expansion leads to the formation of a fibrocartilage upon reimplantation, which is biomechanically inferior to the native hyaline cartilage.

The current culture conditions lead to a concomitant change in both morphology and gene expression profile of articular chondrocytes<sup>124,125</sup>. Specifically, as the cells start to dedifferentiate they flatten to assume a more fibroblast-like phenotype; correspondingly, as the cells flatten their gene expression shifts from a hyaline cartilage phenotype, which is dominated by Col2 expression to a more fibrocartilage phenotype, which is dominated by Col1 expression<sup>124,125</sup>. Therefore, there is a pressing need to improve culture conditions during *in vitro* expansion whereby the cells maintain their morphology and gene expression profile. To address these needs, one strategy involves re-differentiation of the expanded cells through high-density cultures<sup>126</sup>. Although this strategy can restore the morphology of articular chondrocytes there is a paucity of data illustrating that these cells have regained a chondrogenic expression profile. Another common strategy involves supplementation of growth media with growth factors that are important during cartilage



development, such as IGF-1, FGF-2, or TGF $\beta$ 2<sup>127</sup>. Although this strategy leads to moderate restoration of the hyaline-cartilage gene expression profile it is yet to be determined if these factors lead to the synthesis of an ordered ECM around the cells<sup>127</sup>. Therefore, there remains a critical need to develop dynamic culture conditions that expand, enhance their chondrogenic properties, and stimulate the chondrocytes to synthesize and deposit an ordered, hyaline-like matrix.

In healthy articular cartilage, ECM remodeling is initiated by mechanical stimuli that signal to articular chondrocytes through intracellular Ca<sup>2+</sup> waves<sup>48,84,87,128,129</sup>. Studies investigating TRPV4 in articular chondrocytes provide additional support for the importance of Ca<sup>2+</sup> signaling in articular cartilage physiology<sup>130,131</sup>. Specific deletion of TRPV4, an osmo-sensitive Ca<sup>2+</sup> channel, leads to impaired metabolic activity and accelerated onset of degenerative joint disease (OA), highlighting the importance of both TRPV4 and normal Ca<sup>2+</sup> flux in diarthrodial joints<sup>130,131</sup>.

In this study, we explore how the chondrogenic potential of primary bovine articular chondrocytes alters their response to Ca<sup>2+</sup> signaling. To identify the optimal culture conditions for maintaining the chondrogenic properties of articular chondrocytes we cultured the cells in four different media conditions that differ in [Ca<sup>2+</sup>] (1.8mM or .1mM) and the presence/absence of serum. Given the optimal conditions to maintain the differentiation potential we developed a Ca<sup>2+</sup> pulsing protocol to mimic the dynamic mechanical loading experienced in an articulating joint. Taken together, we assessed if the downstream signaling effects of mechanical loading, such as Ca<sup>2+</sup> signaling, are dependent on the differentiation status of articular chondrocytes. Ultimately, the Ca<sup>2+</sup> pulsing protocol we develop in this study can serve as a tool to assess the signaling dynamics of mechanotransduction-relevant pathways of articular chondrocytes; more

importantly, it can also be used to improve upon cartilage reparative techniques, such as ACI.

## **Materials/Methods**

### Cell culture/isolation

Articular cartilage was harvested from bovine knee joints obtained from a local abattoir (JBS USA). Shavings of the articular cartilage were sequentially digested in .2% Pronase (Roche) for 2 hours followed by overnight digestion in .2% collagenase (Gibco), all while shaking at 37°C. Cell suspensions were passed through 70µM cell strainers (BD Falcon) and centrifuged at 500xG for 10 minutes to recover chondrocytes. Chondrocytes were plated at a density of  $1 \times 10^6$  cells/well in 12 well plates and cultured in one of four different media: (1) Dulbecco's minimum essential media (DMEM; Gibco) (1.8mM  $\text{Ca}^{2+}$  + 10%FBS) (2) Low  $\text{Ca}^{2+}$  DMEM (0.1mM  $\text{Ca}^{2+}$  + 10% FBS) (3) Serum-free DMEM (1.8mM  $\text{Ca}^{2+}$  - FBS) (4) Serum-free, Low  $\text{Ca}^{2+}$  DMEM (0.1mM  $\text{Ca}^{2+}$  - FBS) in 6%  $\text{CO}_2$  at 37°C. All media were supplemented with penicillin and streptomycin (Gibco). Serum-free media were supplemented with insulin-transferrin-selenium (Sigma).

### $\text{Ca}^{2+}$ -pulsing regimen

Starting 24 hours post-plating, bovine articular chondrocytes were stimulated with varying concentrations of  $\text{Ca}^{2+}$  (0, 2, 4, 8, 12, or 16 mM total) in the pulsing media (DMEM + ITS) for one treatment period each day. For dynamic  $\text{Ca}^{2+}$  pulsing, cells were treated for 1 hour, recovered in 0.1mM  $\text{Ca}^{2+}$  for 1 hour, and stimulated for 1 hr. with the same [ $\text{Ca}^{2+}$ ] before replacing the original culture media. By contrast, static  $\text{Ca}^{2+}$  stimulation entailed incubation in elevated [ $\text{Ca}^{2+}$ ] for 3 continuous hours before replacing the original culture media.

### RNA isolation/qRT-PCR

RNA was isolated and purified using the Trizol® reagent (Life Technologies) according to the manufacturer's protocol. 1 µg of RNA was converted to cDNA using the SuperScript® III First-Strand Synthesis system (Invitrogen). The cDNA (40ng/reaction) served as the template in the quantitative real-time PCR reaction using ABI-Prism 7300 instrument (Applied Biosystems) using SYBR Green PCR mix (Invitrogen) and specific primers at a final concentration of 70nM. Results were normalized to mRNA levels of

*18srRNA* and *GAPDH*. The primer sequences are as follows: (*Collagen 1* forward:

AATCCAAGGCCAAGAAGCATG, *Collagen 1* reverse: GGTAGCCATTTCCTTGGTGGTT) (*Collagen 2* forward:

CATCTGCTCAGCTGACCTCC, *Collagen 2* reverse: GGGTCCTACGATGTCCTTGAT) (*Collagen 6* forward:

CTGGAGAGCCTGGACAGAAG, *Collagen 6* reverse: GCCTTTGAAACCAGGAACAC) (*Collagen 10* forward:

CCATGCTGGGTAGGTCTGTATAAG, *Collagen 10* reverse: CAGGTAGCCCTTGATGTACTCAT) (*Sox9* forward:

CCGGTGCGCGTCAAC, *Sox9* reverse: GCGCCCACACCATGAAG) (*GAPDH* forward: AATTCTGGCAAAGTGGACATC,

*GAPDH* reverse: GACCATGTAGTGAAGGTCAATGAA) (*18srRNA* forward: TCGAGGCCCTGTAATTGGAA, *18srRNA*

reverse: GCTATTGGAGCTGGAATTACCG).

### Western Blotting/ Dot Blot analysis

Cell lysates were prepared in RIPA buffer with cComplete™ protease inhibitor tablets and phosSTOP™ phosphatase inhibitor tablets (Roche Diagnostics). Protein components were resolved by SDS-PAGE gel electrophoresis, transferred to nitrocellulose membrane, which was then blocked in Tris-buffered saline (LiCor®), sequentially probed with primary and secondary antibodies, and imaged using the LiCor® Odyssey® CLx imaging system. Validated primary antibodies (used at a dilution of 1:1,000) were specific for total CamKII (Abcam, ab134041), T286 phospho-CamKII (Cell signaling technologies, 12716S), β-actin (loading control, LiCor® 926-42212), Col 2 (Abcam, ab21291) and Col 6 (Abcam, ab6588). Fluorescent-labeled anti-rabbit (LiCor®, 925-32211) and anti-mouse secondary antibodies (LiCor®, 925-68070) were used at a

dilution of 1: 5,000. Dot blot analysis was performed as described for Western blotting, except that conditioned media was collected from cell cultures on day 7 was directly spotted on nitrocellulose membrane. Specificity controls for dot blot analysis were rat tail collagen solution (negative control) (Sigma, C3867) and hyaline articular cartilage (positive control) from bovine knee joints. Dot blot signal was quantified using the LiCor® Odyssey® CLx imaging system.

#### Cell viability assay/image acquisition

Chondrocyte viability was assessed using the Live/Dead® viability/cytotoxicity kit (Life Technologies, L3224) immediately following the end of experiments. Images were acquired using a Leica DMI6000B microscope (Leica Microsystems) and analyzed using the Volocity® 3D Image Analysis Software (Perkin-Elmer).

#### Statistical analysis

The error bars in each of the graphs represent the standard error of the means of at least 3 independent experiments, where n= the number of bovine joints. Statistical significance was determined using the Student's t-test with p-values less than .05 considered significant.

### **Results**

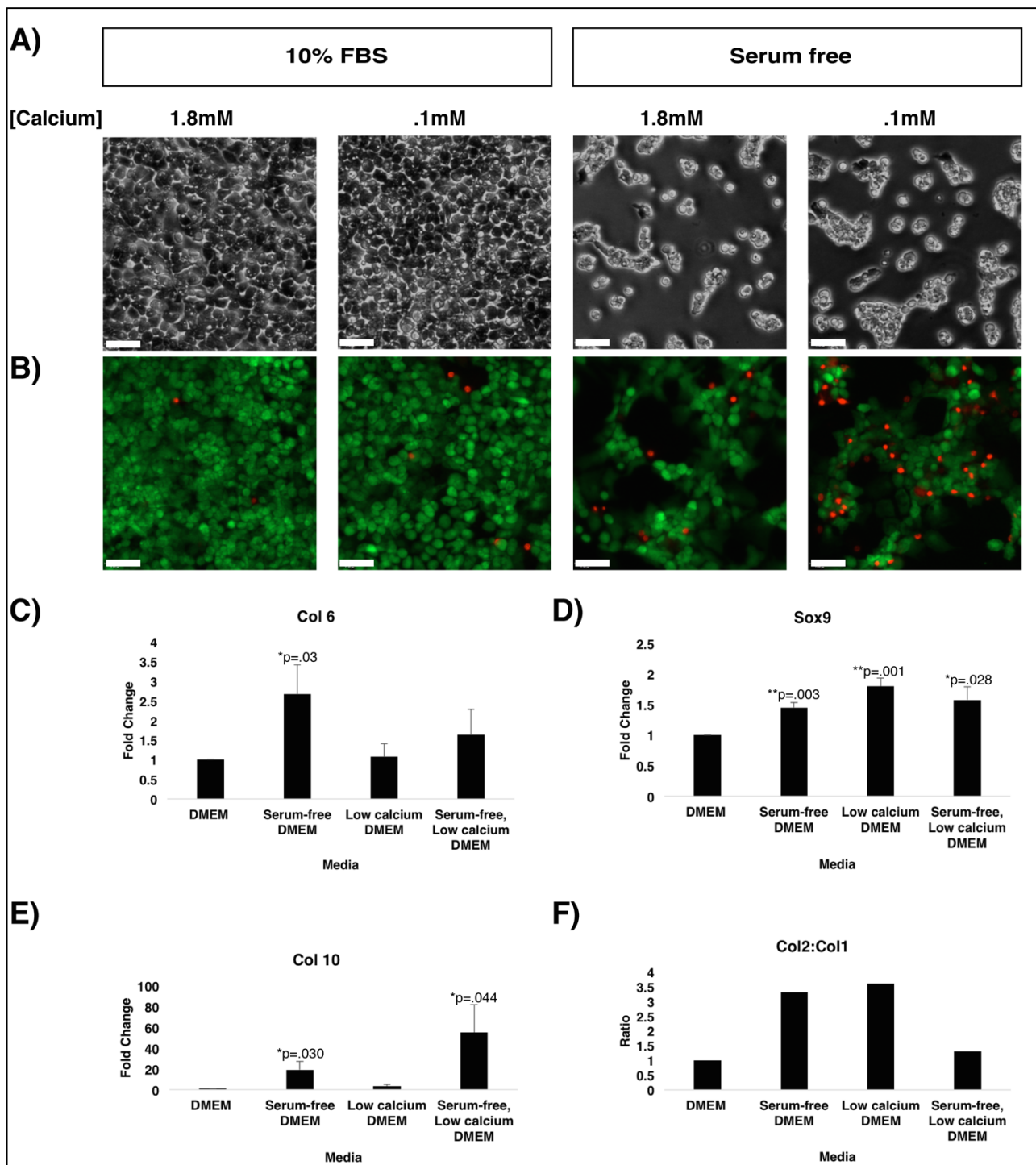
#### Ca<sup>2+</sup> and serum influence differentiation potential of primary bovine articular chondrocytes

A major focus in chondrocyte biology is the changes in ion flux found in extra-cellular matrix during mechanotransduction, most notably changes in Ca<sup>2+</sup> signaling<sup>84,86,132,133</sup>. To understand the importance of Ca<sup>2+</sup> signaling in the differentiation

potential of primary articular chondrocytes we cultured cells in conditions that differ in  $[Ca^{2+}]$ , as well as the presence or absence of serum, which contains  $Ca^{2+}$  buffering agents<sup>134</sup>. We find that cells retain the rounded morphology found *in vivo* in the absence of serum, regardless of  $[Ca^{2+}]$  (*Figure 4a*). Conversely, cells assume a fibroblast-like phenotype shortly after plating down in serum-containing media, illustrating the high rate of dedifferentiation, and less chondrogenic-promoting properties of this media (*Figure 4a*). To ensure that the rounded morphology is not indicative of an apoptotic program, we performed a viability assay on these cells and find that there are slightly more cells that show red fluorescence (dead) than green fluorescence (live) in the serum-free cultures relative to the standard DMEM media. Therefore, cells that are rounded have not undergone apoptosis. (*Live/dead data Figure 4b*) To adequately assess the effects of  $Ca^{2+}$  and serum on the differentiation potential of primary articular chondrocytes we sought to establish the gene expression profile of major chondrogenic genes in the different cultures. We find that there is an increase in *Col VI* (predominant collagen found in pericellular matrix of articular cartilage), and *Sox9* (a major chondrogenic marker) expression in the serum-free cultures relative to the standard DMEM media (*RT-PCR in Figure 4c and 4d, respectively*); however, *Col X* (a well-established hypertrophic marker) is also elevated in the serum free cultures, potentially indicative of the onset of a hypertrophic program (*Figure 4e*). Despite this increase in *Col X* expression, we find that there is an increase in the *Col2:Col1* ratio in the serum-free cells (*Figure 4f*), which is a well-established differentiation index of hyaline cartilage.

**Figure 4 Primary articular chondrocytes maintain their differentiation status in serum-free media.**

(A) Bovine articular chondrocytes were cultured in monolayer for 7 days in 4 different media conditions: DMEM (1.8mM  $[Ca^{2+}]$ , 10%FBS), Serum-free DMEM (1.8mM  $[Ca^{2+}]$ , no FBS), low calcium DMEM (.1mM  $[Ca^{2+}]$ , 10% FBS), Serum-free low calcium DMEM (.1mM  $[Ca^{2+}]$ , no FBS). Scale bar = 22 $\mu$ m. (B) Cell viability analysis of bovine articular chondrocytes following 7 days in monolayer culture in the 4 different media conditions. Live cells are represented through green fluorescence and dead cells are represented through red fluorescence. Scale bar = 21 $\mu$ m. n=3. Gene expression analysis of bovine articular chondrocytes following 7 days in monolayer culture in the 4 different media conditions through qRT-PCR. (C) *Col 6* expression. (D) *Sox 9* expression (E) *Col 10* expression. (F) Ratio of the fold change in expression of *Col 2* to the fold change in expression of *Col 1*. n $\leq$ 4. Error bars represent standard error of the mean. Asterisks and p-values above columns indicate statistically significant differences between the media and basic DMEM media (1.8mM  $[Ca^{2+}]$ , 10%FBS).



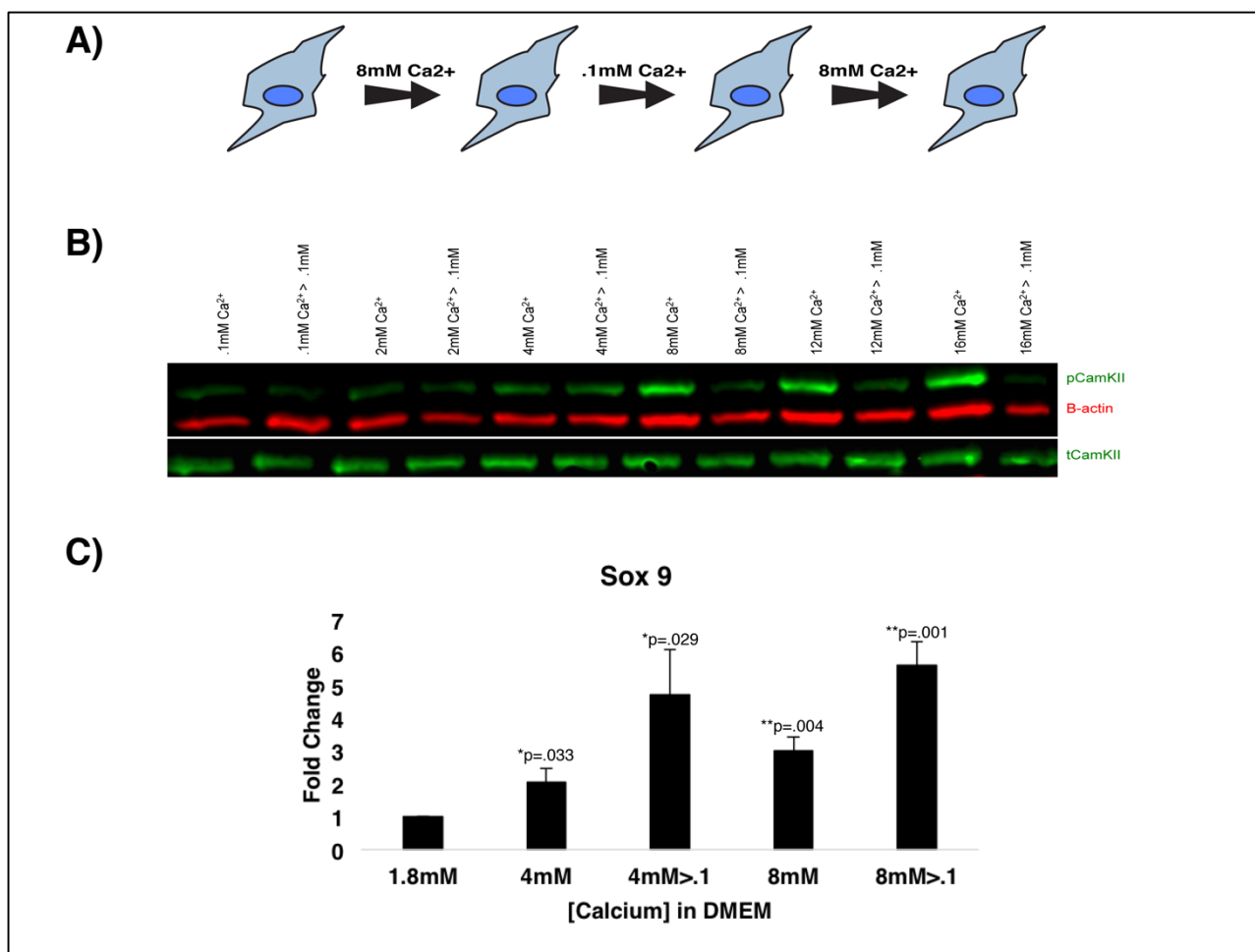
## Primary articular chondrocytes respond robustly to dynamic changes in extracellular $[Ca^{2+}]$

$Ca^{2+}$  signaling is well established as a primary mediator of mechanotransduction in articular chondrocytes<sup>84,87,130</sup>. Moreover, it is known that dynamic mechanical loading leads to a higher chondrogenic potential in articular chondrocytes relative to static mechanical loading<sup>48,132,133</sup>. To directly assess the downstream effects of dynamic mechanical stimuli we stimulated primary bovine articular chondrocytes in a pulsatile manner by alternating between media with high and low  $Ca^{2+}$  concentrations in 1-hour intervals (*Pulsing schematic in Figure 5a*). A well-established downstream mediator of  $Ca^{2+}$  signaling is CamKII, which becomes phosphorylated once the intracellular  $Ca^{2+}$  rises above a threshold, and therefore utilized the phosphorylation of CamKII as a marker of activation of  $Ca^{2+}$  signaling in articular chondrocytes<sup>23,135,136</sup>. We find that in primary articular chondrocytes CamKII is reversibly phosphorylated in a dose-dependent manner; specifically, 8mM of  $Ca^{2+}$  is the minimal concentration that can maximally activate the signaling pathway in a reversible manner (*Figure 5b*). Coordinately, stimulating articular chondrocytes with 4mM and 8mM leads to an increase in *Sox9* expression, paralleling the activation profile of CamKII; however, unlike the dephosphorylation of CamKII, *Sox9* expression continues to increase when the cells are cultured in low  $[Ca^{2+}]$  for one hour (*Figure 5c*).



**Figure 5: Primary articular chondrocytes respond to dynamic changes in extracellular  $\text{Ca}^{2+}$  through upregulation of *Sox9* and activation of  $\text{Ca}^{2+}$  signaling.**

(A) Schematic of the  $\text{Ca}^{2+}$  pulsing regimen, as described in the Methods section, is used throughout the study and was performed daily or following 7 days in culture. Western blot expression analysis (B) of phospho-CamKII (Thr 286), total CamKII, and  $\beta$ -actin (loading control) and gene expression analysis (C) of *Sox9* through qRT-PCR in bovine articular chondrocytes cultured in DMEM for 7 days and following the 7-day culture, stimulated with the indicated  $[\text{Ca}^{2+}]$  for 1 hour with, or without, another hour in low  $[\text{Ca}^{2+}]$  (.1mM). Error bars represent standard error of the mean. Asterisks and *p*-values above columns indicate statistically significant differences between the media and untreated DMEM media. n=3.

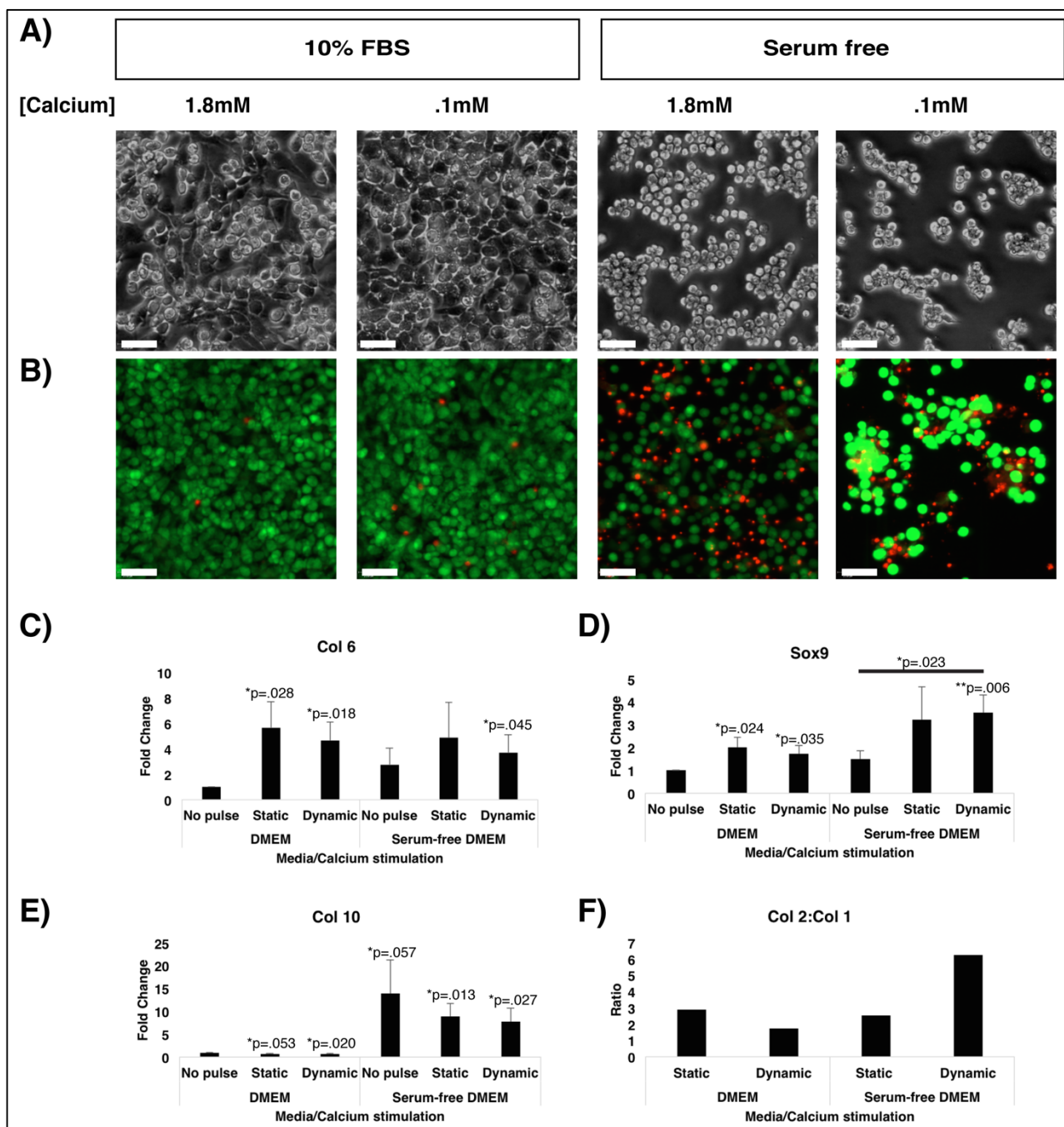


Differentiation potential of articular chondrocytes determines the response to dynamic  $\text{Ca}^{2+}$  signaling.

To determine the functional effect of maintaining differentiation potential of articular chondrocytes in serum-free media we examined the response of articular chondrocytes to daily, dynamic  $\text{Ca}^{2+}$  signaling (two hours of 8mM  $\text{Ca}^{2+}$  stimulation with an hour of recovery in .1mM of  $\text{Ca}^{2+}$  between the stimulations) or static  $\text{Ca}^{2+}$  signaling (three hours of 8mM  $\text{Ca}^{2+}$  stimulation) for 7 days.  $\text{Ca}^{2+}$  pulsing maintained the rounded morphology of cells grown in serum-free cultures, regardless of  $[\text{Ca}^{2+}]$ , and did not drastically affect the morphology of cells in serum-containing media (*Figure 6a*). The cell death profile is unaffected by  $\text{Ca}^{2+}$  pulsing during long-term culture as serum containing cultures continue to have low cell death and serum-free cultures have relatively mildly elevated number of dead cells (*Figure 6b*). When compared to static  $\text{Ca}^{2+}$  signaling and unstimulated controls we find that dynamic  $\text{Ca}^{2+}$  signaling leads to an enhancement of the chondrogenic expression profile through increases in *Col VI* and *Sox9* expression levels (*Figure 6c and 6d*). While there is an increase in the *Col X* expression levels in the serum-free media, we find that  $\text{Ca}^{2+}$  stimulation in both types of media leads to a decrease in *Col X* expression levels (*Figure 6e*). The chondrogenic enhancement of  $\text{Ca}^{2+}$  pulsing is further illustrated in the increase in Col2:Col1 ratio; interestingly however, we find that the response to  $\text{Ca}^{2+}$  stimulation is dependent on the culturing media. In serum-free media, the more chondrogenic media, we find that cells have a higher Col2:Col1 ratio in response to dynamic  $\text{Ca}^{2+}$  signaling, whereas cells cultured in the presence of serum have a higher Col2:Col1 ratio in response to static  $\text{Ca}^{2+}$  stimulation (*Figure 6f*).

**Figure 6: Cells cultured in serum-free media respond to dynamic stimulation with  $\text{Ca}^{2+}$  through upregulation of chondrogenic genes.**

(A) Bovine articular chondrocytes cultured in the 4 different media conditions and  $\text{Ca}^{2+}$ -pulsed daily for 7 days according to the schematic presented in figure 2. Scale bar =  $22\mu\text{m}$ . (B) Cell viability analysis of bovine articular chondrocytes cultured in the 4 different media conditions and  $\text{Ca}^{2+}$ -pulsed daily for 7 days. Scale bar =  $21\mu\text{m}$ .  $n=3$ . Gene expression analysis of bovine articular chondrocytes cultured in DMEM and serum-free DMEM and  $\text{Ca}^{2+}$ -pulsed daily for 7 days either statically (3 consecutive hours of  $8\text{mM Ca}^{2+}$ ) or dynamically (according to the pulsing schematic in figure 2) through qRT-PCR for (C) *Col 6* expression, (D) *Sox 9* expression, (E) *Col 10* expression, (F) Ratio of the fold change in expression of *Col 2* to the fold change in expression of *Col 1*.  $n=5$ . Error bars represent standard error of the mean. Asterisks and  $p$ -values above columns indicate statistically significant differences between the treatment and basic DMEM media ( $1.8\text{mM [Ca}^{2+}\text{]}$ ,  $10\%\text{FBS}$ ).



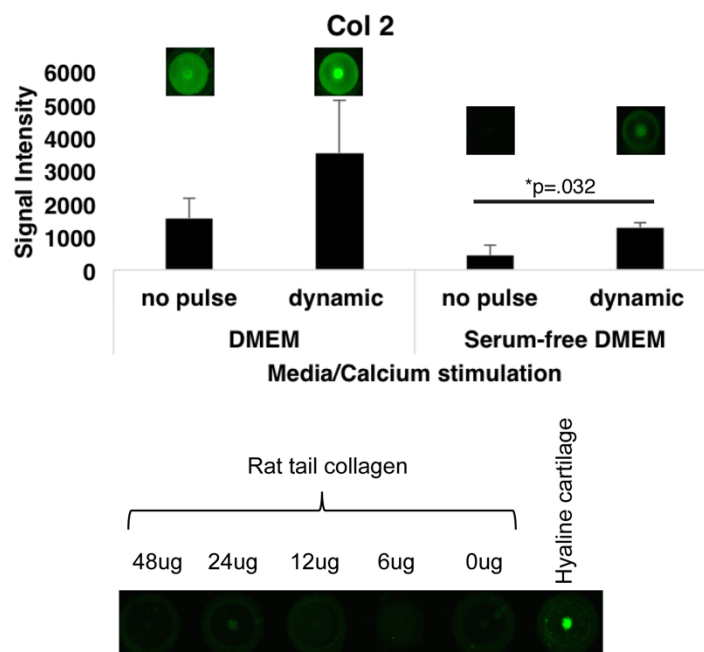
Dynamic  $\text{Ca}^{2+}$  stimulation of differentiated primary articular chondrocytes leads to the secretion of hyaline cartilage constituents.

To assess if dynamic  $\text{Ca}^{2+}$  signaling can enhance the production of hyaline cartilage constituents, such as collagen 2 and collagen 6, we performed a dot blot analysis of the culturing media following 7 days of daily  $\text{Ca}^{2+}$  pulsing. Similar to our gene expression data, we find that the production of Col 2 and Col 6 are enhanced by dynamic  $\text{Ca}^{2+}$  signaling in the more chondrogenic serum-free media; interestingly however, we find that cells cultured in the presence of serum respond to dynamic  $\text{Ca}^{2+}$  signaling by decreasing the production of Collagen 6 (*Figure 7a and 7b*).

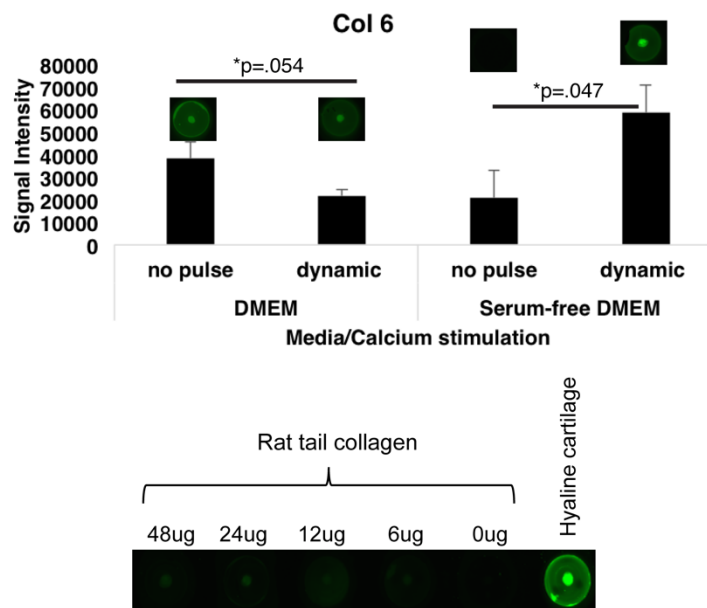
**Figure 7: Dynamic stimulation of primary articular chondrocytes with  $\text{Ca}^{2+}$  leads to the secretion of hyaline cartilage constituents.**

Dot blot analysis of (A) Col 2 and (B) Col 6 in bovine articular chondrocytes cultured in DMEM and serum-free DMEM and dynamically  $\text{Ca}^{2+}$ -pulsed for 7 days. The images above columns are representative of the signals visualized on the dot blot membrane for each of the media and treatment groups. Rat collagen tail, which predominantly contains col 1, serves a negative control and bovine hyaline cartilage, which predominantly contains col 2 and col 6, serves as a positive control.  $n=3$ . Error bars represent standard error of the mean. Asterisks and  $p$ -values above columns indicate statistically significant differences.

A)



B)





## Discussion

Cartilage homeostasis is maintained through extracellular matrix remodeling by articular chondrocytes, which are stimulated through dynamic mechanical loading<sup>36,115,137,133</sup>. It is well established from previous studies that mechanical forces result in intracellular  $\text{Ca}^{2+}$  waves in articular chondrocytes and the induction of ECM components occurs through dynamic signaling processes, rather than a static stimulation<sup>87,132,138</sup>. Many studies have used varying modes of mechanical stimulation to enhance the chondrogenic properties of cartilage constructs; however, these studies overlook the differentiation potential of the chondrocytes seeded in the constructs<sup>48,84,114,132,137</sup>. If  $\text{Ca}^{2+}$  signaling is critical for cartilage homeostasis, we hypothesized that the maintenance of chondrogenic properties in isolated articular chondrocytes is dependent on  $[\text{Ca}^{2+}]$  during *in vitro* culture. In this study, we have established that the response to dynamic  $\text{Ca}^{2+}$  signaling by articular chondrocytes serves as a novel differentiation index, while enhancing the chondrogenic properties of articular chondrocytes. Taken together, this study addresses a critical unmet need to improve upon the quality of cells used for ACI procedures; additionally, it advances and continues the discussion on the importance of  $\text{Ca}^{2+}$  signaling in chondrocyte biology for cartilage homeostasis and osteoarthritis progression.

We first sought to establish the culture conditions that would maintain the differentiation status and morphology of chondrocytes found *in vivo*. We cultured the cells in different media varying in  $[\text{Ca}^{2+}]$  and in the presence or absence of serum. In serum-free conditions the cells retain a more rounded morphology reminiscent of cells found *in vivo*; on the other hand, cells cultured in the presence of serum assume a flattened fibroblast-like morphology, indicative of dedifferentiation (*Figure 4a*). It is important to note that the rounded morphology in the serum-free conditions does not automatically indicate

the initiation of an apoptotic program; while there are more dead cells in serum-free conditions, they do not represent the majority of the cell population (*Figure 4b*). Importantly, the cells cultured in serum-free conditions, relative to those cultured in standard DMEM media, exhibit a gene expression profile that reflects that of hyaline cartilage. Specifically, they have low expression of *Col I* and increased expression of *Col II*, *Col VI*, and *Sox9*. This profile leads to a remarkably high Col II: Col I ratio, a defining feature of hyaline cartilage. In addition, the serum-free conditions lead to a high expression of *Col X*, a marker of hypertrophic chondrocytes, which we have been able to suppress with the addition of soluble factors without losing the hyaline cartilage phenotype (*data not shown*). These data led us to conclude that the optimal media to retain the differentiation status of articular chondrocytes is that which does not contain any serum. Surprisingly, we did not see any significant differences in morphology or gene expression between the two serum-free media conditions that differ in  $[Ca^{2+}]$  (1.8mM and .1mM of  $Ca^{2+}$ ). Similarly, there is a mild effect of solely lowering the  $[Ca^{2+}]$  in the presence of serum, indicating that the critical dedifferentiation factor, which remains to be determined, is contained in the serum. Therefore, our results corroborate the notion that articular chondrocytes retain their differentiation status in serum-free conditions as they are housed in a metabolically inert microenvironment *in vivo*.

The dedifferentiation of cells during ACI leads to the production of fibrocartilage upon reimplantation to repair chondral lesions; however, fibrocartilage is biomechanically inferior to the native hyaline cartilage<sup>109,122,123</sup>. Therefore, in addition to maintaining the differentiation status of chondrocytes, it is important to have culture conditions that stimulate cells to secrete matrix components reminiscent of hyaline cartilage. Several groups have illustrated that stimulating  $Ca^{2+}$  signaling pathways through the TRPV4

channels has resulted in the upregulation of hyaline cartilage components<sup>130,131</sup>. However, a major caveat to these methods of analyzing  $\text{Ca}^{2+}$  signaling is the static nature of the stimulation, as numerous studies have illustrated the upregulation of matrix components in articular chondrocytes occurs through dynamic stimuli<sup>86,132</sup>. As a result, we sought to assay the response of articular chondrocytes to dynamic  $\text{Ca}^{2+}$  signaling through the development of a novel protocol whereby we expose the cells to high and low concentrations of  $\text{Ca}^{2+}$  in a pulsatile manner (*Figure 5a*). A critical mediator of  $\text{Ca}^{2+}$  signaling in neurons and cardiomyocytes, both physiologically and pathologically, is CamKII, which is expressed in both growth plate chondrocytes and articular chondrocytes<sup>23,135,139</sup>. As in other systems, we find that there is a critical threshold of  $\text{Ca}^{2+}$  concentration that leads to a reversible phosphorylation of the critical Thr286 residue of CamKII (*Figure 5b*). This data indicates that the articular chondrocytes are viable and responsive to  $\text{Ca}^{2+}$  concentrations as high as 16mM. We find that the response to an increase in  $\text{Ca}^{2+}$  is not dose-dependent as increasing the extracellular concentration of  $\text{Ca}^{2+}$  does not enhance the expression of *Sox9*; nor is it dependent on the phosphorylation status of CamKII, as one hour in low extracellular concentration of  $\text{Ca}^{2+}$  dephosphorylates CamKII but further increases the expression of *Sox9* levels (*Figure 5c*). This could be explained by the residual activation of downstream transcription factors controlling the expression of chondrogenic genes despite the removal of a stimulus<sup>140,141</sup>. These results provide rationale for further studies on the temporal and spatial dynamics of  $\text{Ca}^{2+}$  signaling in articular chondrocytes.

The utility of dynamic  $\text{Ca}^{2+}$  signaling lies in its ability to enhance the chondrogenic potential of articular chondrocytes<sup>130,132,138</sup>. In this study, we have shown that compared to static  $\text{Ca}^{2+}$  signaling and unstimulated controls dynamic  $\text{Ca}^{2+}$  signaling leads to an

enhancement of the chondrogenic properties of articular chondrocytes through the chondrogenic gene expression profile (*Figure 6c and 6d*). Furthermore, the cells cultured in the absence of serum respond to dynamic  $\text{Ca}^{2+}$  signaling by releasing hyaline cartilage constituents, further supporting the chondrogenic-enhancing properties of dynamic  $\text{Ca}^{2+}$  signaling (*Figure 7*). This differential response to  $\text{Ca}^{2+}$  stimulation of cells based on the culturing media led us to conclude that the differentiation status of articular chondrocytes plays a critical role in how cells respond to stimuli. We show in this study that cells cultured in serum-free media, the more chondrogenic media, have a higher Col2:Col1 ratio in response to dynamic  $\text{Ca}^{2+}$  signaling, whereas cells cultured in the presence of serum have a higher Col2:Col1 ratio in response to static  $\text{Ca}^{2+}$  stimulation (*Figure 6f*). In other words, cells that have maintained their differentiation potential respond to mechanical stimulation with different signaling dynamics than cells that have dedifferentiated. This finding is critical for the field of chondrocyte biology and tissue engineering, because it informs us that the downstream effect of mechanical stimulation is highly dependent on the underlying biology of the cell. Therefore, future studies will aim to further delineate if the differential responses to  $\text{Ca}^{2+}$  signaling correlate to the progression of normal cartilage to diseased cartilage.

In conclusion, our study addresses an unmet need to enhance the chondrogenic potential of articular chondrocytes for ACI. We have developed a novel assay that determines the differentiation potential of chondrocytes, which may in turn be used in tissue engineering applications to seed scaffolds with highly chondrogenic cells. In line with our work, others assert that  $\text{Ca}^{2+}$  signaling is vital in mediating mechanotransduction signals to articular chondrocyte, however, there are other groups that maintain that  $\text{Ca}^{2+}$  signaling negatively regulates chondrogenesis<sup>131</sup>. Our study firmly establishes that  $\text{Ca}^{2+}$

stimulation regulates expression of important components of matrix remodeling. More importantly, however, in contrast to previous studies that have statically stimulated cells, we illustrate the benefit of dynamic  $\text{Ca}^{2+}$  signaling in improving the chondrogenic nature of articular chondrocytes<sup>130</sup>.

Therefore, this study further highlights the importance of  $\text{Ca}^{2+}$  signaling in chondrocyte biology for homeostasis, cartilage tissue engineering, and OA disease progression. Future work will explore the mechanism by which dynamic  $\text{Ca}^{2+}$  signaling mediates matrix remodeling, and in turn how matrix remodeling affects the ability of cells to respond to dynamic  $\text{Ca}^{2+}$  signaling. It would be prudent then to investigate if steady dynamic  $\text{Ca}^{2+}$  signaling or aberrant  $\text{Ca}^{2+}$  signaling underlie the difference between chondrocytes found in normal articular cartilage and those found in arthritic cartilage. In summary, this provides a novel means to address important questions in chondrocyte biology that will lead to a better understanding of OA onset.

**Chapter 3: RNA-sequencing analysis of bovine articular chondrocytes.**

## Introduction

The articular chondrocyte is a highly-specialized cell that resides in an extremely dynamic environment. Its developmental history can be traced from the interzone of the developing synovial joint and the margins of the cartilage anlagen during joint cavitation to the mechanosensitive surfaces of a growing postnatal articular cartilage<sup>7,12</sup>. As the long bone grows and the growth plate closes, the articular cartilage thickness gradually decreases from its postnatal girth encasing the articular chondrocyte in an aneural, avascular, hypoxic environment<sup>27</sup>. This environment renders the articular chondrocyte quiescent for most of its lifecycle so that it does not expend the metabolic resources that are in scarcity<sup>142</sup>. The only exception occurs upon joint loading, which stimulates the cells through mechanotransduction pathways, such as  $\text{Ca}^{2+}$  signaling, to remodel the extracellular matrix to maintain cartilage homeostasis<sup>143,144</sup>. Any aberration to the structure of articular cartilage, therefore, directly influences stimulation of articular chondrocytes and their subsequent response.

The response of articular chondrocytes to stimuli is critical because it underlies the principles of using autologous cells to repair cartilage lesions in younger patients<sup>107,123</sup>. The ability of autologous cells to fill the defect with hyaline cartilage components has improved functionality of knee joints in many patients that have incurred traumatic joint injury. To improve upon these outcomes, a modification to this procedure entails implanting a collagen-based scaffold embedded with the cells into the defect site<sup>114,115</sup>. This modification was introduced because various studies have implicated embedding chondrocytes into a three-dimensional matrix to mimic the *in vivo* environment to maintain and enhance the chondrogenic potential of articular chondrocytes<sup>145–148</sup>. The chondrogenic potential of articular chondrocytes can be defined

as the gene expression signature defining articular chondrocytes and the capacity to synthesize hyaline cartilage components. A major unmet need in the field of articular chondrocyte biology is defining the culture systems and the expression signatures that can identify and maintain a stable articular chondrocyte phenotype for downstream tissue engineering and orthopedic surgery applications.

In the previous chapter, we observed that culture condition-altered differentiation potential of articular chondrocytes influenced response to  $\text{Ca}^{2+}$  signaling. We observed that cells in serum-free cultures display significantly higher Col2:Col1 ratio (*Figure 4f*), a well-established differentiation index, than serum-containing cultures. In this study, we set out to further test the effects of culture conditions on the transcriptional profile of articular chondrocytes by taking cells from a single joint and growing them under three conditions: (1) a dedifferentiated two-dimensional monolayer culture, (2) a three-dimensional alginate hydrogel culture to maintain chondrogenic phenotype, and (3) a culture condition to test redifferentiation potential by embedding seven-day monolayer-cultured cells into three-dimensional hydrogels for seven days<sup>126,148,149</sup>. We hypothesized that the cells in each of the different culture conditions would exhibit vastly different gene expression profiles. Additionally, we found that chondrocytes maintained in serum-free medium, but not in serum containing medium, upregulated chondrogenic genes, such as *Sox9* (*Figure 6D*) and secreted hyaline cartilage constituents, such as Col2 and Col6 (*Figure 7*) in response to dynamic (pulsatile)  $\text{Ca}^{2+}$  stimulation. However, there is a discordance between traditional expression signatures in growth media (DMEM). As the synthesis of hyaline cartilage components and the ratio of *Col2:Col1* decreases upon stimulation, *Sox9* continues to increase (*Figure 6D, 6F*). This could potentially be explained by the uncoupling of *Sox9* regulation of Collagen 2 upon dedifferentiation;



however, it does not explain the decrease in synthesis of Col6 as well<sup>150</sup> (*Figure 7b*). To understand the full breadth of differentiation potential requires examination of the genetic expression profile, rather than just the transcriptional profile of a few genes. We hypothesized that an expansion of genes we are looking at could explain the discordance found in the previous study. To attain a thorough gene expression signature of the different culture conditions we decided to perform an in-depth transcriptional analysis through RNA-sequencing, which utilizes deep-sequencing technologies to provide a panorama of the transcriptional landscape<sup>151–154</sup>.

In combination with culture conditions, we tested the effects of age on the gene expression signature of articular chondrocytes. It is well known that age is a risk factor for the development of osteoarthritis<sup>105,155–157</sup><sup>158</sup>. Structurally, with age there is degradation of collagen fibrils and changes in composition of the side chain sulfate groups of proteoglycans, which give rise to coordinate changes in water composition<sup>41</sup><sup>159,160</sup>. In addition to these structural changes, aging cartilage has been shown to contain higher concentrations of inflammatory cytokines, reactive oxygen species, and mediators of autophagy; furthermore, human articular chondrocytes exhibit a decline in response to growth factor signaling with age<sup>161–166</sup><sup>167</sup>. This disparity in chondrocyte biology with age convinced a group to test the use of juvenile cadaveric allogeneic chondrocytes for ACI and found that cells from younger patients exhibited a higher chondrogenic profile<sup>117</sup>. Similarly, RNA-sequencing analysis of two different age groups of equine articular chondrocytes showed almost 400 transcribed elements were differentially expressed between the two age groups<sup>168</sup>.

In this study, we explore the effects of interdependency of age and culture condition on the gene expression signature of articular chondrocytes. The central

hypothesis of this study is that younger chondrocytes would be able to redifferentiate and have a tighter expression profile. Whereas older chondrocytes would be unable to redifferentiate and exhibit properties similar across all culture conditions with greater genetic diversity. To test this hypothesis, we cultured articular chondrocytes from young (< 30 months of age) and old bovine joints (>40 months of age) in culture conditions representing three differentiation states: (1) a dedifferentiated two-dimensional monolayer culture, (2) chondrogenic three-dimensional alginate hydrogel culture, and (3) a redifferentiated state in which monolayer-cultured cells were subsequently embedded in alginate hydrogels following seven days. Through RNA-sequencing analysis, we find that culture conditions have a significantly greater influence on the gene expression profile of articular chondrocytes than age; however, we find that older cells have a tighter expression profile than younger cells, especially in the redifferentiated cultures. Additionally, we find that there is a discordance between overall gene expression profile and the major chondrogenic genes, which are not significantly differentially expressed between culture conditions or age groups; thereby casting doubt on their sensitivity as markers of articular chondrocyte differentiation. The drastic influence of culture conditions, as revealed by the deep sequencing analysis in this study, will put the spotlight back on identifying the facets of culture conditions that are critical for maintaining the chondrogenic phenotype; however, the analysis also reveals that it may be time to reexamine what genes and properties we use to define a chondrogenic phenotype for articular chondrocytes.

## **Materials/Methods**

### Sample collection/Histology

A local abattoir (JBS, USA) supplies bovine knee joints from two age groups that are classified as young and old: (1) < 30 months of age, but no less than 20 months of age and (2) > 40 months of age. Articular cartilage is shaved and sequentially digested, while shaking, in .2% Pronase (Roche) for 2 hours at 37° followed by overnight digestion in .2% collagenase (Gibco) at 37°. The cells are separated from the matrix by gravity filtration through 70µM cell strainers (BD Falcon). From the same joint, full thickness cartilage biopsies are harvested using a 6mm punch biopsy (Integra™ Miltex™, 3336) and immediately fixed in 4% paraformaldehyde (© 2017 Alfa Aesar, Thermo Fisher Scientific, 30525-89-4) in PBS overnight for downstream histological analysis of proteoglycans and collagens through safranin-O and alcian blue staining, respectively. The cells were isolated from 4 different young joints (n=4) and 3 different old joints (n=3).

### Cell culture

Following separation, the cells are washed and then pelleted by centrifugation at 500xG for 10 minutes. To establish isogenic controls, cells from the same joint are cultured in either a two-dimensional monolayer culture for fourteen days or embedded within a three-dimensional sodium alginate bead for fourteen days. The cells in two-dimensional monolayer culture are maintained in Dulbecco's minimum essential media (Invitrogen, 11965-092) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin-glutamine (Invitrogen, 10378-016), and Amphotericin B (Gibco, 15290026). The cells in three-dimensional alginate bead cultures are maintained in DMEM/F12 (1:1) media supplemented with 1% penicillin-streptomycin-glutamine (Invitrogen, 10378-016),

Amphotericin B (Gibco, 15290026), insulin-transferrin-sodium selenite (Sigma, I2771), 50 $\mu$ g/mL Vitamin C, 10ng/mL FGF2, and 10ng/mL TGF- $\beta$ 3 (PeproTech®, 100-36E).

Redifferentiated cells were those that were cultured in two-dimensional monolayer culture for 7 days and then embedded in three-dimensional alginate bead cultures for 7 days, with each phase the cells are cultured in the respective media.

### Alginate encapsulation and RNA isolation

A 1.5% weight/volume alginate solution was made by dissolving PRONOVA™ Ultra-pure medium viscosity sodium alginate, with guluronate constituting 60% of the monomer units (Novamatrix®, ©2016 FMC 4200106), in 1x PBS and sterile-filtered. A final concentration of about 75 million cells per mL was determined to be optimal in the fourteen-day alginate cultures and about 20 million cells per mL in the redifferentiated cultures. Individually, each aliquot of cells was pelleted, the media was aspirated, and the cell pellet was resuspended in 1.5% alginate solution made with PBS (sterile-filtered). The alginate-cell suspension was mixed carefully with a combination of pipetting and stirring to avoid accidental lysis of cells due to shear forces from the viscous alginate solution. The cell-alginate suspension was loaded into a 3mL syringe with a 22G needle and extruded dropwise into 50mM CaCl<sub>2</sub>/140mM NaCl for polymerization of the beads. The beads were incubated for about 2 minutes in the polymerization solution before being transferred to a separate well and washed twice in DMEM/F12 media prior to placement in culture media (DMEM/F12 +ITS +Vitamin C + 10ng/mL FGF2 +10ng/mL TGF $\beta$ 3). RNA was isolated and purified using the Trizol® reagent (Life Technologies) according to the manufacturer's protocol. RNA concentrations were determined using Thermo Scientific™ NanoDrop™ and RNA quality was determined on the Agilent Bioanalyzer 2100 (© Agilent technologies).

## RNA-sequencing analysis: cDNA library preparation, sequencing, and alignment of reads

cDNA libraries were prepared from 250ng of RNA using the reagents of the TruSeq RNA kit from Illumina® on the SciClone® workstation from PerkinElmer, Inc. Quality check of libraries was performed using DNA-1000 kit on the Agilent Bioanalyzer 2100 ((© Agilent technologies). The concentration of each of the final libraries was determined using ThermoScientific™ Qubit™. We multiplexed 24 libraries, which were diluted, pooled together, and denatured with .2N sodium hydroxide. The final concentration of the libraries that were carefully loaded onto the sequencer was 1.3nM. We performed a 150 single-base pair read MidOutput run on the NextSeq 500 machine. The data is presented as Fastq files that are demultiplexed to create .bam files which are run through the standard pipelines utilizing STAR as the aligner and RSEM as the tool for annotation and quantification at both gene and isoform levels.

## Statistical analysis and Ingenuity pathway analysis

RNA-seq data were quantified and normalized in Transcripts Per Kilobase Million (TPM) values, and available for 24596 genes. Multidimensional scaling plot of distances between gene expressions profiles were used to examine the relationship of samples. Differential expression analysis was conducted using the limma package in R<sup>169–171</sup>. A multi-level linear model was fitted to the expression data accounting for correlation between measurements made on the same cattle<sup>171</sup>. The false discovery rate (FDR) was controlled globally using the Benjamini and Hochberg algorithm<sup>172</sup>. Genes with FDR < 0.05 and fold-change > 2 were judged to be differentially expressed. The datasets were uploaded into Ingenuity® Pathway Analysis (IPA®) tool (© Qiagen Bioinformatics) and

core and comparison analysis were performed on all datasets to identify canonical pathways and molecules that are differentially regulated.

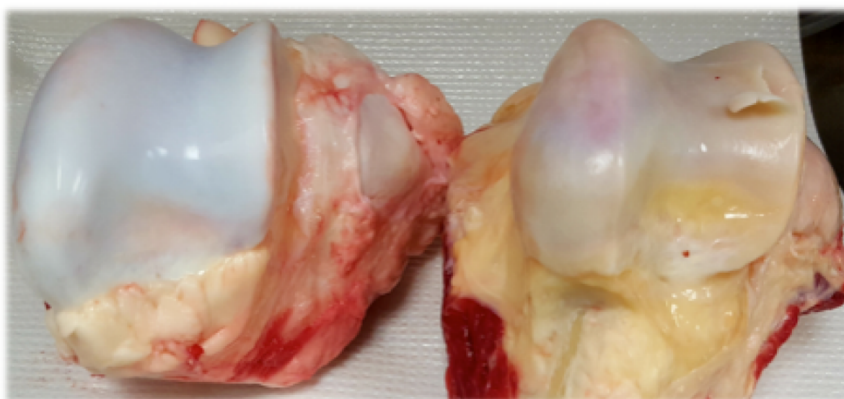
## Results

### Younger and older bovine articular cartilage exhibit histological differences in proteoglycan content and collagen organization.

One of the hallmarks of aging articular cartilage is the gradual loss of proteoglycans and collagens and the infiltration of water; additionally, in older joints you can visualize macroscopic changes such as fibrillation and increased adiposity<sup>66,104,159</sup>. The joints that we harvested cells and cartilage biopsies from exhibited most, if not all, of these differences. The joint on the left is from a young cow, as evidenced by the pearly-white covering of articular cartilage and the low level of fat that is deposited in the peri-articular area (*Figure 8A*). Conversely, the joint on the right is from an older cow, as evidenced by the fibrillations on the articular surface, the loss of the shiny white gloss, and the increase in adiposity in the peri-articular area. The changes in proteoglycan and collagen content are commensurate with the macroscopic changes, as there is greater safranin-o and alcian blue staining, respectively, in the younger joint relative to the older joint (*Figure 8B*). Similarly, there is a graded decrease in proteoglycan content as the distance from the lesion site decreases in the older joint (*Figure 8C*). These results illustrate that there are significant macroscopic and ultrastructural changes that are occurring with age and at cartilage lesion sites.

**Figure 8: Younger and older bovine articular cartilage exhibit histological differences in proteoglycan content and collagen organization.**

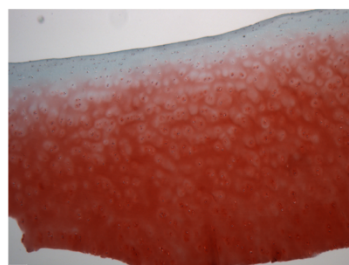
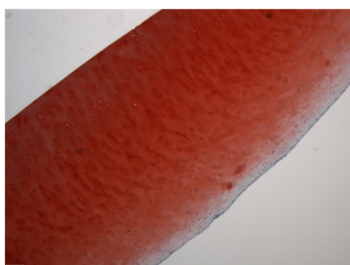
- (A) Photograph of the young (< 30 month of age, but no less than 20 month of age) bovine knee joint is shown on the left, while the old (> 40 months of age) bovine knee joint is shown on the right.
- (B) Safranin-O staining was performed to assess proteoglycan content and alcian blue staining was performed to assess collagen content of articular cartilage from young and old bovine knee joints.
- (C) Safranin-O staining was performed to assess proteoglycan content of articular cartilage at various distances from the site of a lesion.

**A)****B)**

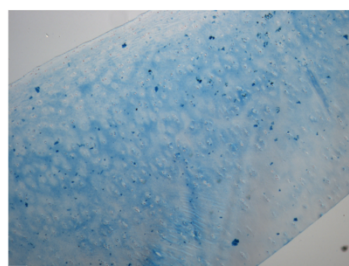
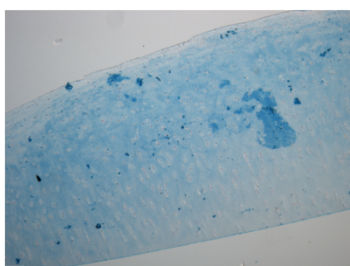
Young BAC

Old BAC

Safranin-O



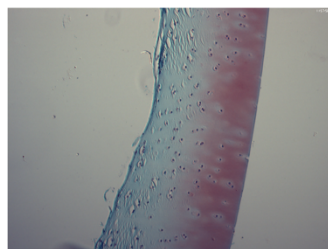
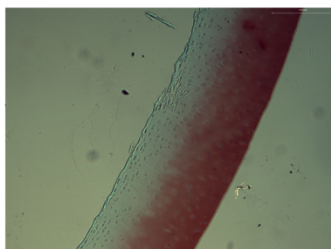
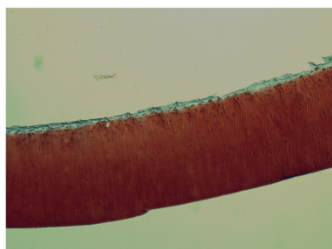
Alcian Blue

**C)**

Unaffected

Intermediate

Lesion





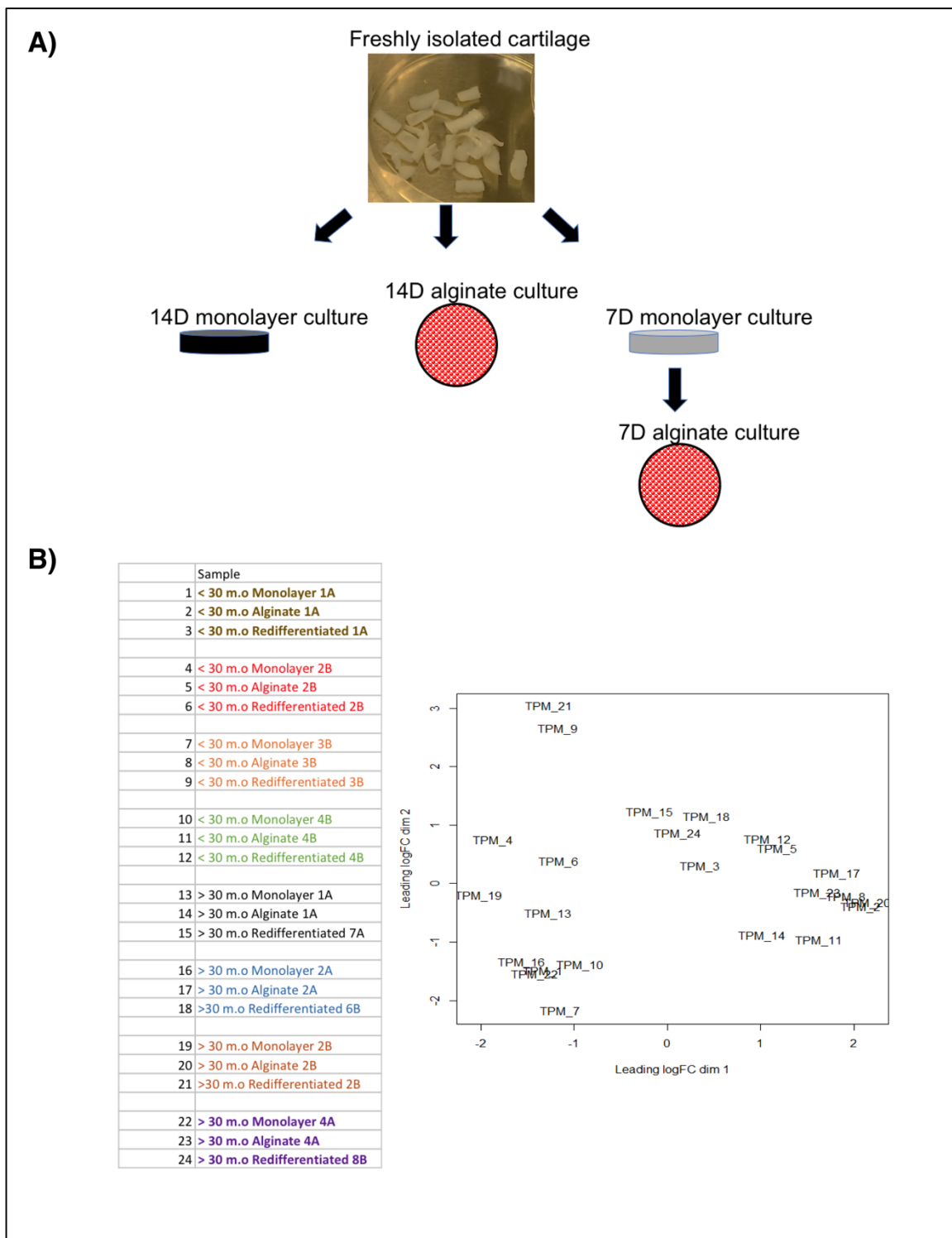
Culture conditions influence the gene expression profile of articular chondrocytes more than age.

Given that expansion of articular chondrocytes *in vitro* leads to dedifferentiation, groups have sought to embed cells in a three-dimensional scaffold to maintain their phenotype<sup>126,149,173,174</sup>. To this end, orthopedic surgeons have combined collagen-based scaffolds with autologous chondrocytes in a procedure known as matrix-assisted autologous chondrocyte implantation (*Figure 3*). When examining the long-term efficacy of these procedures it was determined that juvenile cadaveric allogeneic cells maintained a hyaline phenotype far greater than those from autologous cells, indicating that age is a critical factor in cell quality<sup>116,117</sup>. In this study, we tested the interdependency of age and culture condition on the gene expression profile by culturing young and old bovine articular chondrocytes in two-dimensional monolayer culture, three-dimensional alginate culture, and tested the redifferentiation capacity of the cells by culturing in monolayer for seven days and embedding in alginate culture for seven days (*Figure 9a*). When examining the relationship of gene expression between samples using the multidimensional scaling plot, we find that samples clustered together based on culture condition (*Figure 9b*). For instance, regardless of age all the monolayer-cultured samples cluster in the lower left quadrant; whereas, all the alginate-cultured samples cluster in the lower right quadrant (*Figure 9b*). Intriguingly, all the cells that underwent redifferentiation clustered in between the monolayer and alginate-cultured samples; however, there was greater scattering of the redifferentiated samples than the other two culture conditions. These results indicate that culture conditions have a greater influence on gene expression profile than age; additionally, the variability in the

redifferentiation process is illustrated by the increased scattering of gene expression profiles of cells that were embedded in alginate culture following dedifferentiation.

**Figure 9: Culture conditions influence the gene expression profile of articular chondrocytes more than age.**

- (A) Schematic of experimental design defining the different culture conditions for RNA-sequencing
- (B) Multidimensional scaling plot of distances between gene expression profiles to examine the relationship between samples. List of the 24 RNA samples submitted for RNA sequencing analysis. The number in the plot corresponds to the number of the sample.  $n=4$  in the young population and  $n=3$  in the older population.

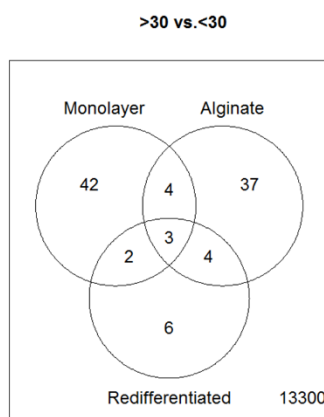
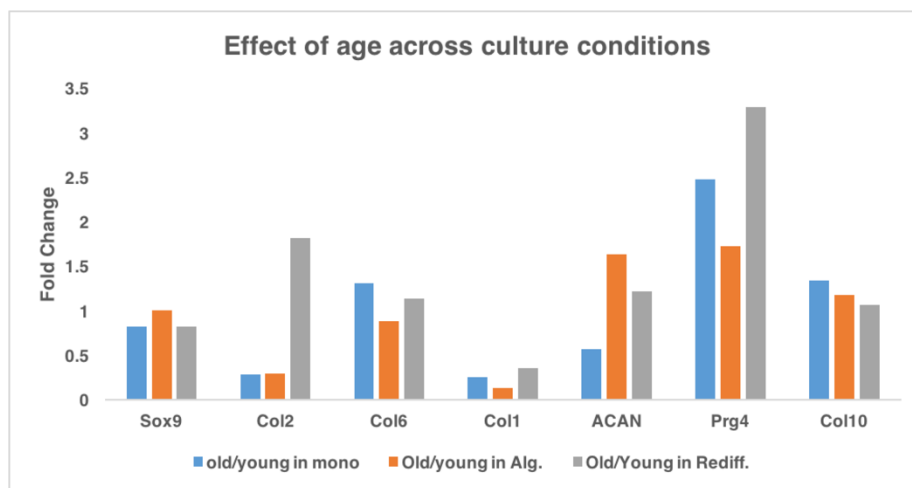


There is greater genetic diversity among younger chondrocytes than older chondrocytes.

While clustering was based on culture conditions, a closer examination of the samples illustrates that within a given culture condition there are subtle differences between the two age groups. When comparing the age groups in monolayer, alginate, and redifferentiated cultures there are 51, 48, and 15 differentially expressed genes between the two age groups (*Figure 10a*). The major chondrogenic genes however are not significantly differentially expressed between the age groups in any of the three culture conditions (*Figure 10b*). Upon reexamination of the multidimensional scaling plot it is appreciated that the expression profiles of younger samples cluster together within the monolayer and alginate culture conditions (*Figure 9b*). In contrast, the expression profiles of older cells cluster together upon redifferentiation more than the younger cells, potentially indicating greater transcriptional lability in the younger cells. These results illustrate that within culture conditions there are differences in expression profile between the two age groups as evidenced by the differentially expressed genes as well as the differential clustering, although the gene expressions that are significantly different do not include major chondrogenic genes.

**Figure 10: Differential expression analysis of age in the various culture conditions.**

- (A) Venn diagram detailing the number of differentially expressed genes in an older joint relative to a younger joint in the three different culture conditions.
- (B) Fold change of the major chondrogenic genes in an older joint relative to a younger joint in the three different culture conditions.

**A)****B)**

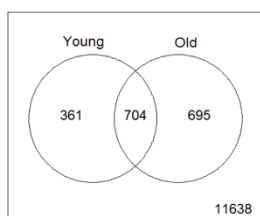
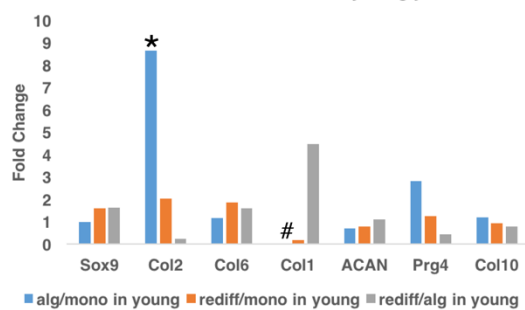
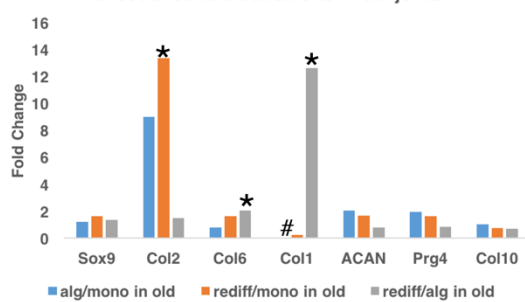
Traditional expression signatures of articular chondrocytes do not corroborate the culture condition-induced diversity of expression profiles.

The distinct expression profiles between the samples based on culture conditions is reflected in the number of differentially expressed genes between culture conditions within an age group. Within the young chondrocytes there are 2824 genes differentially expressed between the Monolayer and Alginate\_groups, 1065 genes differentially expressed between the Monolayer and Redifferentiated groups, and 2771 genes were differentially expressed between the Alginate and Redifferentiated groups (*Figure 11a*). Similarly, within the older chondrocytes there are 2313 genes differentially expressed between the Monolayer and Alginate groups, 1399 genes differentially expressed between the Monolayer and Redifferentiated groups, and 2563 genes differentially expressed between the Alginate and Redifferentiated groups (*Figure 11a*). The higher number of differentially expressed genes between culture conditions within age groups, relative to the number of differentially expressed genes between age groups within culture conditions, agrees with the clustering of the gene expression profiles according to culture conditions. Once again, however, we do not find that the major chondrogenic genes are significantly differentially expressed between the culture conditions, with the exception being that Col1 is downregulated consistently in alginate cultures relative to monolayer cultures, regardless of age (*Figure 11b*). These results demonstrate that gene expression profiles are highly-dependent on the culture conditions; however, the major chondrogenic genes are not contributing to these vast differences in expression profile, which is in stark contrast to the studies in cartilage and chondrocyte biology that use these genes as sensitive markers of differentiation.



**Figure 11: Differential expression analysis of the various culture conditions between the two age groups.**

- (A) Venn diagram detailing the number of differentially expressed genes between the three different culture conditions within the two age groups.
- (B) Fold change of the major chondrogenic genes between the three different culture conditions within the two age groups. Asterisks denote significant upregulation and pounds denote significant downregulation. Genes with a False Discovery Rate (FDR)  $< .05$  and fold change  $> 2$  were labeled as significant.

**A)****Alginate vs. Monolayer****Redifferentiated vs. Monolayer****Redifferentiated vs. Alginate****B)****Effect of culture conditions in young joints****Effect of culture conditions in old joints**

## Discussion

Orthopedic surgeons employ autologous chondrocyte implantation for repair of focal cartilage lesions<sup>109</sup>. A critical factor in the long-term efficacy of these procedure is the cell quality of the reimplanted cells<sup>116</sup>. Cell quality remains a key challenge for ACI because *in vitro* expansion of autologous chondrocytes leads to dedifferentiation, which promotes formation of biochemically and biomechanically inferior fibrocartilage upon reimplantation. Thus, there is an unmet need to define the culture systems and expression signatures that can identify and maintain a stable articular chondrocyte phenotype. Studies have shown that embedding chondrocytes in a three-dimensional scaffold maintains a stable phenotype, and subsequently has been adopted as a modification to ACI, whereby cells are implanted with a collagen scaffold<sup>118,119</sup>. Additionally, when juvenile cadaveric chondrocytes are used as a source of cells there was an improvement in the measures of chondrogenic potential for ACI, thereby establishing that age is a critical factor in chondrocyte quality<sup>117</sup>.

In the previous chapter, we established that that culture condition-induced alterations in differentiation potential influence the response of articular chondrocytes to  $\text{Ca}^{2+}$  signaling, a primary mediator of mechanotransduction; intriguingly, however, we find that not all the major chondrogenic genes respond coordinately to dynamic  $\text{Ca}^{2+}$  signaling raising questions about the specificity and sensitivity of the markers that we use to classify chondrogenic articular chondrocytes<sup>83</sup>. To address these questions, in this chapter we utilized the deep sequencing technology of RNA sequencing to explore how the gene expression signature of articular chondrocytes changes across different culture conditions and across two age groups (*Figure 8a, 9a*); additionally, it allows us to address the efficacy of using conventional chondrogenic markers to define articular

chondrocytes<sup>152,175,176</sup>. Taking the results of the RNA sequencing and incorporating it into a multidimensional scaling plot allowed us to examine the relationship of the gene expression profile of the twenty-four samples to each other (*Figure 9b*). We found that samples from the same culture conditions clustered closer together than samples from the same age. Two-dimensional monolayer-cultured samples clustered far away from three-dimensional alginate cultured samples with the redifferentiated samples clustering in between the two. These results support the commonly-held hypothesis that two-dimensional cultures of articular chondrocytes have a distinct genetic profile from three-dimensional cultures of articular chondrocytes. This is the first study to perform an in-depth expression profile of cells that have undergone redifferentiation, and while our hypothesis was supported by the intermediary relationship of the redifferentiated samples with the other two culture conditions, it was intriguing to find that the redifferentiated samples exhibited greater scattering in expression profiles relative to the other two culture conditions. This result reveals that there is a great deal of genetic diversity in the fate of cells that have dedifferentiated and undergone redifferentiation, calling into question the exact nature of the identity of these cells. Upon closer examination of the redifferentiated samples, we find that samples from older joints clustered tighter together, indicating a more similar expression profile, than samples from younger joints, which contrasts with what we anticipated. This suggests that there is greater transcriptional lability in the younger samples than in the older samples allowing for a more diverse genetic expression profile upon redifferentiation. A possible mechanistic explanation for this differential response to redifferentiation based on age may lie in the accrual of epigenetic events that lead to tighter epigenetic regulation in the older samples, thereby resulting in a constraint on the diversity of genetic fates upon redifferentiation. To test this hypothesis would require the removal of epigenetic marks

from older samples and examine if the expression profiles parallel the younger samples upon redifferentiation. Nonetheless, these results illustrate that the cells undergoing redifferentiation have great genetic heterogeneity than we first anticipated, explaining the variability in long-term efficacy of autologous chondrocyte implantation.

To identify the efficacy of exclusively using chondrogenic markers to define articular chondrocytes we examined if they were included in the differentially expressed genes among all the samples. We find that when comparing the age groups in monolayer, alginate, and redifferentiated cultures there are 51, 48, and 15 differentially expressed genes between the two age groups (*Figure 10a*). The number of differentially expressed genes warrants the conclusion that the two age groups have different expression profiles; however, the chondrogenic genes would refute this conclusion because they are not significantly differentially expressed between the two age groups in any of the three culture conditions (*Figure 10b*). On the other hand, the number of differentially expressed genes between culture conditions within an age group is forty times more than the number of differentially expressed genes when comparing age groups within a culture condition (*Figure 11a*). This result corroborates the distribution of the samples on the multidimensional scaling plot, once again illustrating that the influence of culture conditions on the gene expression profile vastly outweighs the influence of age. Once again, however, we do not find that the major chondrogenic genes are significantly differentially expressed between the culture conditions, with the exception being that Col1 is downregulated consistently in alginate cultures relative to monolayer cultures, regardless of age (*Figure 11b*). These results cast further doubt regarding the reliability of using the major cartilage genes to understand the chondrogenic potential of articular chondrocytes. One possible explanation for these

results is that the normalization of the raw read counts of transcripts for the generation of the TPM values may mask the changes in expression that would otherwise be identified; as a result, the statistically significant changes in the chondrogenic genes observed in chapter 2 provide important biological information because expression was assessed using qRT-PCR<sup>151,175,177–179</sup>. Despite the modest changes in the chondrogenic genes the high number of differentially expressed genes across the culture conditions regardless of age illustrate the prodigious influence culture conditions have on the differentiation potential of articular chondrocytes.

This is the first study to examine the effects of interdependency of age and culture conditions on the gene expression profile of articular chondrocytes. The isogenic controls in the study ensure that the differences in expression profiles observed between culture conditions are not due to whole genome variations. Similarly, histological data from the same joints as the cells from which RNA was harvested for RNA-sequencing analysis helps correlate expression changes to macroscopic changes. Therefore, the experimental design provides confidence in the conclusion that regardless of age, culture conditions have a far greater influence on the expression profile of articular chondrocytes. The organization of the expression profiles with the redifferentiated cells falling in between the two-dimensional and three-dimensional cultures indicates that the genetic identity of the cells used for cartilage resurfacing procedure is unknown, as the cells are often dedifferentiated *in vitro* for expansion. An important question that stems from these studies is how can we modify the redifferentiation process to push the gene expression profile closer to the three-dimensional cultures, or even better towards the expression profile of native articular cartilage, which will be determined in future studies. This question can be addressed by modulating the temporal profile of the culture

conditions, as well as by modulating the soluble factors that are supplemented. These answers will allow us to modify the process by which we culture autologous chondrocytes prior to reimplantation to ensure the highest cell quality to improve upon the long-term efficacy of cartilage resurfacing procedures. However, the utility of the chondrogenic genes in ensuring the highest cell quality remains in doubt. One means to address this issue would be to identify the differentially expressed genes within the samples by utilizing the raw transcript counts. If this method of analysis revealed that the major chondrogenic genes were included in the significantly differentially expressed genes then it would reveal that there is a distinct layer of transcriptional regulation for the chondrogenic genes that is being normalized during standard RNA-sequencing analysis. If, however, the chondrogenic genes are shown to not be significantly differentially expressed, despite altering the method of analysis, then it would open a plethora of questions regarding why these markers of chondrogenic differentiation remain unchanged across culture conditions that have clearly distinct expression profiles. Furthermore, it would beg the question as to what expression signature would define an articular chondrocyte? Thus, the conclusions of this study are important for cell biologists, tissue engineers, and orthopedic surgeons alike because it would lead to a shift in how we define, modulate, and apply articular chondrocytes for enhancing the cartilage restorative process.

## **Chapter 4: Discussion**



## **Developmental history highlights intersection of articular cartilage homeostasis and degeneration**

Articular cartilage, a metabolically and macroscopically inert tissue, works with ligaments, muscles, and synovial fluid to stabilize, strengthen, and lubricate diarthrodial joints, respectively<sup>1,36</sup>. This coordinated activity of all tissue types allows for painless, smooth locomotion and activity. There is a steady decline in the patency and function of articular cartilage with age, trauma to the joint, or other inciting metabolic and inflammatory agents<sup>128</sup>. The terminal condition of articular cartilage is its degeneration down to the subchondral bone plate of the long bone which it covers<sup>36</sup>. This full-thickness degeneration is a hallmark of the classical condition osteoarthritis, which is also characterized by excessive inflammation and a disruption in synovial joint homeostasis<sup>180,181</sup>.

End-stage osteoarthritis necessitates total joint replacement due to the excruciating pain that accompanies daily activity<sup>182,183</sup>. Prior to joint replacement, orthopedic surgeons have sought to resurface smaller cartilage lesions utilizing biological and tissue engineering principles<sup>115</sup>. These procedures, which will be discussed later in this chapter, aim to replace or regenerate cartilage lesions that often arise in the younger patient population following a traumatic joint injury, such as meniscal or ligament tears<sup>184,185</sup>. Traumatic injuries to the supporting structures of the joint destabilize the joint and result in an uneven distribution of forces on the articular surface<sup>106,186,187</sup>. The altered joint mechanics gradually give rise to the small cartilage lesions that further exacerbate joint homeostasis with the terminal condition being osteoarthritis and a need to replace the entire articular surface.

Joint mechanics are important because mechanotransduction pathways are the primary means by which cartilage homeostasis is maintained. In a healthy joint, articular cartilage can convert mechanical signals into efficient extracellular matrix remodeling<sup>62,96</sup>. In the immediate aftermath of a joint injury there is a sharp change in activity of articular cartilage due to an increase in extracellular matrix remodeling, but this burst of activity gradually normalizes within weeks<sup>143</sup>. It's known, however, that individuals that have injuries that destabilize joints, such as meniscal or ligament tears, have a 10% increased risk of developing post-traumatic osteoarthritis and eventually necessitating joint replacement<sup>106,188–191</sup>. **The central question that follows then is what gives rise to the progressive decline in articular cartilage homeostasis?** The answer to this question will explain the biological mechanisms that lie at the intersection of cartilage homeostasis and degeneration. Additionally, addressing this question will aid in developing strategies to delay or prevent the development of full-thickness cartilage lesions, a potentially multi-billion-dollar relief for healthcare expenditure.

Understanding the development of synovial joints has shed light on the fundamental mechanisms that direct embryonic and postnatal articular cartilage growth and maturation. There are three critical phases of synovial joint development: (1) Mesenchymal stem cell condensation, (2) interzone cell formation, and (3) joint cavitation<sup>2,3,4</sup>. While the cellular condensation dictates the temporal and spatial origins of the synovial joint, the origin of cartilage anlagen can be traced back to the interzone cells<sup>6,7</sup>. The expression profile and location within the layer of interzone cells distinguishes the progenitors of permanent from the progenitors of temporary cartilage. Following commitment to a specific lineage, mechanotransduction-mediated extracellular matrix remodeling is the primary driving force behind joint cavitation<sup>16–21</sup>. Like growth

plate cartilage, the differentiation and maturation of articular cartilage continues postnatally, however, there are multiple models to explain articular cartilage maturation<sup>23-25</sup>. The differential growth models discussed in Chapter 1 illustrate that there is still debate regarding the metabolic profile of the tissue. If the postnatal growth is mediated by proliferation there must be initiating and terminating signals for due to the paucity of metabolic resources to support a constantly proliferating tissue<sup>26</sup>. Conversely, the interstitial growth model hinges on efficacious extracellular matrix remodeling as a means of increasing tissue growth<sup>33</sup>. This model has yet to resolve, however, the temporal and spatial details of the signals that direct the breakdown and synthesis of the extracellular matrix. It follows then that articular cartilage developmental dynamics may parallel the mechanisms of cartilage homeostasis in mature tissue.

In the fully mature articular cartilage, cartilage homeostasis relies on the interdependence of extracellular matrix composition and articular chondrocyte matrix remodeling. As highlighted in Chapter 1 articular chondrocytes respond to mechanical and chemical changes in the pericellular matrix as an initial step in the remodeling of the extracellular matrix<sup>45,192</sup>. Additionally, the gradual change in relative proportions of articular cartilage composition with mechanical, chemical, and biological insults play a critical role in how chondrocytes receive and respond to stimuli. It follows then, would modification of the extracellular matrix be sufficient to restore cartilage homeostasis following traumatic joint injury? Or would injection of mesenchymal stem cells into relatively healthy extracellular matrix be sufficient for the cells to efficiently differentiate into adult chondrocytes for subsequent repair of cartilage lesions? Furthermore, recent studies have evidence that following injury, cells migrate towards the defect site to initiate a repair response, which begs the questions is it sufficient to introduce

chemotactic agents into an injured joint to potentiate a repair response through this mechanism<sup>193</sup>?

Surgical procedures to resurface joints in the young, active patient were also highlighted in chapter 1. The four main procedures reviewed were microfracture surgery, ACI/MACI, and AMIC<sup>115,119</sup>. All procedures aim to replace a degenerating site of cartilage with an autologous, marrow, or stem cell population complemented with or without a three-dimensional scaffold to maintain the cellular differentiation potential<sup>115,194</sup>. The successes and shortcomings of these procedures highlight the ingenuity of collaborations between biologists, tissue engineers, and orthopedic surgeons, while drawing attention to problems whose solutions will improve long-term efficacy. There are two critical unmet needs with all procedures highlighted by recent studies: (1) improving quality of implanted cells and (2) properties of implanted scaffolds. A recent study illustrated that cell quality affects the long-term efficacy of ACI's, which was addressed by using allogeneic juvenile articular chondrocytes by another group, which raises the question how do articular chondrocytes change with age<sup>116,117</sup>? Another strategy to address hypertrophy and dedifferentiation of implanted cells is the engineering of cartilage constructs with scaffolds from varying biological sources, such as chitosan<sup>118</sup>. The development of cartilage constructs in the field of tissue engineering raises the question, how do articular chondrocytes modulate their transcriptional profile when forced to be in environments that alter their differentiation potential? The studies detailed in chapters 2 and 3 address these questions by illustrating the coordinated relationship between transcriptional profile and differentiation potential with varying extracellular matrix composition and age groups.

## **Differentiation potential of chondrocytes dictates response to loading events**

My studies began with exploring how the chondrogenic potential of primary bovine articular chondrocytes alters their response to  $\text{Ca}^{2+}$  signaling. Given that articular chondrocytes reside in an avascular, aneural, and nutrient-deficient environment *in vivo* we can modulate the differentiation potential of cells by culturing them in either serum-containing (dedifferentiating) or serum-free (chondrogenic) media (*Figure 4*). Serum-free cultures display significantly higher Col2:Col1 ratio, a well-established differentiation index, than serum-containing cultures. To simulate a joint loading event the cells were treated with elevated  $[\text{Ca}^{2+}]$  for up to three hours that elicited activation of downstream mechanosensitive pathways. We find that chondrocytes maintained in serum-free medium, but not in serum containing medium, upregulated chondrogenic genes (e.g. *Sox9*) and secreted hyaline cartilage constituents (e.g. Col6 and Col2) in response to dynamic (pulsatile)  $\text{Ca}^{2+}$  stimulation (*Figure 6*). As a result, chondrocytes that have maintained their differentiation potential respond to stimuli with different signaling dynamics than cells that have dedifferentiated. This finding is critical for the field of chondrocyte biology and tissue engineering, because it informs us that the downstream effect of mechanical stimulation is highly dependent on the differentiation status of cells, which is known to be variable in the various resurfacing procedures. These results begin to address the central question by positing that the differentiation status of articular chondrocytes is critical for maintaining cartilage homeostasis; additionally, this study introduces that calcium signaling dynamics must be tightly regulated for maintenance and enhancement of chondrogenesis. To this end, we can improve upon the study by utilizing tools, such as optogenetics, to spatially and temporally activate calcium

signaling to attain greater control of the system<sup>195–198</sup>. Precise control of the system would allow us to ask questions about how calcium signaling affects the transcriptional dynamics, thereby providing a mechanistic link between changes in calcium signaling and differentiation status of articular chondrocytes. Therefore, while this study illustrates that calcium signaling helps us determine the chondrogenic potential of articular chondrocytes, it also opens the possibility of asking what are other means of assessing the differentiation potential of articular chondrocytes?

### **Redifferentiation of articular chondrocytes sheds light on transcriptional lability**

The studies in Chapter 2 illustrated that we can modulate the differentiation potential of the cells by altering the culture media. Embedding the cells in a hydrogel scaffold, such as alginate, is another means to modulate the differentiation potential<sup>145,148,149</sup>. Additionally, recent clinical studies have illustrated that the age of the cells used for resurfacing procedures affects the long-term outcomes<sup>117</sup>. Given the importance of culture conditions and age of the cells on the differentiation potential of articular chondrocytes, we asked what effect the interdependency of age and culture conditions have on the expression profiles of articular chondrocytes in chapter 3. We find that culture condition-dependent alterations in transcriptional profile of bovine articular chondrocytes are independent of age (*Figure 9*). However, there is greater genetic diversity among younger chondrocytes than among older chondrocytes upon redifferentiation. Additionally, we find that traditional expression signatures of articular chondrocytes do not corroborate the culture condition-induced diversity of expression profiles (*Figure 10, Figure 11*). The prodigious influence of culture conditions illustrates that the continually remodeled extracellular matrix with age and joint injury *in vivo*, which

macroscopically produce gradual changes that can be repaired, there is a fundamental alteration in the gene expression profile of the resident articular chondrocytes.

Furthermore, the tight expression profile of older chondrocytes, relative to younger chondrocytes, upon redifferentiation indicates that aging factors are preventing articular chondrocytes from transcriptionally adapting to a changing extracellular environment.

While it is uncertain what the genetic profile of the redifferentiated cells are, this genetic diversity illustrates the potential for this cell type to be used in synthetic biology.

Modulation of temporal profiles of culture conditions, modulation of the stiffness or composition of the hydrogels, and modulation of the soluble factors utilized would provide identify the factors contributing to the change in gene expression profile of articular cartilage from a healthy joint all the way to the articular cartilage that lines a degenerating joint.

### **Probing the transcriptional networks of articular chondrocytes through synthetic biology**

The studies presented in this dissertation show that the changing extracellular matrix in an aging joint alters the transcriptional profile of articular chondrocytes through changes in signaling dynamics, such as  $\text{Ca}^{2+}$  signaling. However, there is a lot that needs to be parsed in terms of articular chondrocyte biology from healthy, post-traumatic, and arthritic cartilage. Across these stages, we anticipate that changes in lipid metabolism, autophagy, senescence-associated pathways, and metabolic reprogramming directly shift the transcriptional profile of articular chondrocytes<sup>100,199–201,202</sup>. I believe that we can understand these transcriptional states using synthetic biology to engineer transcriptional networks and asking how specific modulation of signaling pathways affect chondrocyte biology<sup>203–206</sup>. Ultimately, I believe that we must

ask ourselves the question, how does the state of the articular chondrocyte affect the ability of articular cartilage to function? The end goal would be to have the capability to reprogram or enhance articular chondrocytes in coordination with the changing joint biology.



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